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The Actomyosin ATPase Inhibitory Region of Troponin I: Synthetic Studies on the Relationship of Primary Structure to Biological Function.

University — Université

Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Ph.D.

Year this degree conferred — Année d'obtention de ce grade

1981

Name of Supervisor — Nom du directeur de thèse

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Date Feb 17/81

DEDICATION

"When you've hit a really tough one, tried everything, racked your brain and nothing works, and you know that this time Nature has decided to be difficult, you say, "Okay, Nature, that's the end of the nice guy," and you crank up the formal scientific method."

Zen and the Art of Motorcycle
Maintenance by Robert M. Pirsig

To my parents, brothers, sister and Guy Lafleur. Probably in that order.

ABSTRACT

Previous studies have shown that a large part of the actomyosin ATPase inhibitory activity of troponin-I (Tn-I) was located in one of its cyanogen bromide fragments (residues 96-116). The most notable feature of this fragment was the number of basic residues it contained. We have synthesized peptides containing this sequence using the solid phase technique. The inhibitory activity of these peptides and Tn-I was greatly enhanced by the presence of tropomyosin. Conversely, salmine, a representative small basic protein, which inhibited the actomyosin ATPase in the absence of tropomyosin, inhibited less well in the presence of tropomyosin. This proved that the inhibition of our peptides more closely resembled the specific inhibition of Tn-I than the non-specific inhibition of salmine. The peptides also resembled Tn-I in their effect on the binding of tropomyosin by actin and their additional inhibitory effect on actomyosin ATPase. At ionic conditions where the actin was not saturated with tropomyosin the peptides promoted further binding of tropomyosin. At ionic conditions where the actin was saturated with tropomyosin the peptides were capable of causing additional actomyosin ATPase inhibition of their own.

Analogs of varying lengths were constructed to determine the minimum length of the sequence responsible for the inhibitory properties of the cyanogen bromide fragment and by extension Tn-I. Peptides containing the sequence (105-114) showed no significant reduction in inhibitory activity. Further shortening resulted in a substantial loss of inhibitory activity demonstrating the importance of the charged residue lysine 105, and the hydrophobic residue 114, to the

inhibition. The charges on the α-amino and α-carboxyl groups of the peptides, which do not appear in the parent protein, were shown to modify the inhibitory activity of the peptides. If either a positive or negative terminal charge was close to the minimum inhibitory region a large decrease in activity resulted.

The active peptides were 45% as active as whole Tn-I on a molar basis. When actin and tropomyosin were proportionately increased in the actomyosin ATPase assay, the ratio of inhibitor (both Tn-I and peptides) to actin required to achieve full inhibition was decreased.

Our peptides thus possessed the ability to mimic Tn-I isolated from fast skeletal muscle. This suggested that the behaviour of Tn-I from other sources might be explained by differences in their amino acid sequence in the inhibitory region. Our precisely determined inhibitory region proved that the differences in inhibitory activity of Tn-I isolated from rabbit fast and rabbit slow skeletal muscle were not the result of differences in this inhibitory region. Their sequences in this region were identical. The only known sequence of Tn-I that differed in the region homologous to 105-114 of rabbit fast skeletal Tn-I was rabbit cardiac Tn-I. Previous work has shown that rabbit cardiac Tn-I inhibited more poorly than rabbit fast skeletal Tn-I in a rabbit fast skeletal actomyosin system. Peptide analogs of the inhibitory regions of the two species of Tn-I exhibited the same behaviour and allowed us to verify that the loss of arginine 113 was solely responsible for the loss of inhibitory activity of the cardiac analog peptides. This loss of inhibitory activity presumably contributes to the lesser inhibitory activity of rabbit cardiac Tn-I. In summary the importance of the residues mentioned (lysine 105, arginine

113, valine 114) suggests that the site that rabbit fast skeletal Tn-I binds at, contains two carboxyl groups and a hydrophobic pocket.

Bovine cardiac Tn-I has been shown in prior studies to inhibit better than rabbit fast skeletal Tn-I in a bovine cardiac actomyosin system. In contrast rabbit cardiac peptide analogs inhibited less well than rabbit fast skeletal Tn-I analogs in a rabbit cardiac actomyosin system. Further investigation revealed that rabbit cardiac Tn-I inhibited less well than rabbit fast skeletal Tn-I in a rabbit cardiac actomyosin system. Therefore the behaviour of rabbit and bovine cardiac Tn-I relative to rabbit fast skeletal Tn-I in their respective cardiac actomyosin systems was not the same. In total then, both rabbit fast skeletal and cardiac Tn-I peptide analogs mimicked the activity of their parent proteins extremely well.

ACKNOWLEDGEMENTS

It is impossible for me to thank everyone who has been of assistance to me during this project but it would be unpardonable for me not to attempt it. My supervisor, Dr. Hodges has served as a guide, instructor, sounding board, advisor, model and friend during the time I have spent here and I thank him very much.

The members of the M.R.C. group have all been very helpful and unfailingly generous in allowing me to use their labs, equipment and personnel. Drs. Kay and Smillie gave me much friendly assistance, advice and sympathy when things went wrong. Bill McCubbin, Joyce Pearlstone, Peter Johnson, Bill Lewis, Alan Mak, Bill Wolodko and John Shriver all gave me great assistance in understanding the literature and my own results. The assistance, especially in technical matters, of Kim Oikawa, Vic Ledsham, Mike Carpenter, Mike Natriss, Lois Serink, Tony Keri and Krystina Golosinka was always expert, cheerful and patient. Graham Cote, Mary Pato, Dave Johnson, Brian McDonough, Max Hincke, Dave Byers, Gordon Stewart, Lana Lee, Judy Shelling, Maureen O'Connor and Gary Brayer, fellow graduate students, were always supportive, stimulating and great persons with whom to work.

I would like expecially to thank the people I have worked closely with in the lab of Dr. Hodges. Any merit that can be found in this study is in large part due to them. Eric Tong, Donna Clare, Serge St. Pierre, Tjoeng Foe-Siong, Ron Reid, Jean Gariepy, Amrit Saund and Pele Chong have made possible my success because of their help, encouragement and friendship. Much expert help in preparing the thesis was given by Ken Burns, Roger Bradley, Jaclyn Dorsey and Darleen Wilson.

Finally, I would like to thank Drs. Colter, Scraba (thanks for the centrifuge), Bridger, Sykes and James all of whom allowed me to invade their labs and abuse their personnel, even though some of them don't like muscle research. The financial assistance of the Medical Research Council and University of Alberta is gratefully acknowledged and was tremendously appreciated.

TABLE OF CONTENTS

	Page
DEDICATION	IV
ABSTRACT	V
ACKNOWLEDGEMENTS	VIII
LIST OF TABLES	XIV
LIST OF FIGURES	XV
LIST OF ABBREVIATIONS	XVII
CHAPTER I: INTRODUCTION	
A. MUSCLE	1
1. Ultrastructure	2
a. Sarcoplasm	3
b. Sarcomere and the sliding filament	3
c. Sarcolemma, transverse tubules and the sarcoplasmic reticulum	6
2. Infrastructure	9
a. Thick filament	9
b. Thin filament	11
i. Actin	11
ii. Tropomyosin	12
iii. Troponin	12
iv. Thin filament protein interactions	14
v. Two site model	16
vi. Lin Yu-tang and the thin filament	19
c. Rigor complex	21
3. Short Summary of Events in Muscle Contraction	22

Page

4. Inhibition of Actomyosin ATPase 23

a. Troponin-I 23

b. Basic proteins 24

c. Tn-I fragments 24

B. SOLID PHASE PEPTIDE SYNTHESIS 25

C. PURPOSE OF THIS STUDY 27

CHAPTER II: EXPERIMENTAL PROCEDURES

A. SOURCES OF CHEMICALS AND SOLVENTS 30

B. AMINO ACID ANALYSIS, PEPTIDE AND PROTEIN QUANTITATION . . 31

C. EQUIPMENT 31

D. PROTEIN PURIFICATION 31

1. General 31

a. Cautions 31

b. SDS gel electrophoresis 34

2. Actin 34

a. Acetone powder 34

b. Actin 36

3. Myosin 37

a. Myosin 37

b. S-1 41

4. Troponin 44

5. Troponin-C 45

6. Troponin-I 46

E. ACTOMYOSIN AND ACTO-S-1 ASSAYS 49

1. Assay of Inhibitory Activity 49

Page

a. Tn-I 49

b. Peptides 51

2. Phosphate Determination 51

F. PEPTIDE SYNTHESIS AND PURIFICATION 52

1. Attachment to the Solid Support 52

2. Substitution Level Strategy 53

3. Synthesis 53

4. Cleavage of Peptides from Resin 54

5. Purification of Peptides 55

6. Resin Hydrolysis 55

G. F-ACTIN BINDING AND CENTRIFUGATION 56

CHAPTER III: INITIAL CHARACTERIZATION

A. CHEMICAL CHARACTERIZATION 57

1. Synthetic Strategy 57

2. Peptide Purification 63

3. Peptide Yields 65

B. BIOLOGICAL CHARACTERIZATION 70

1. Actomyosin ATPase Inhibition 70

2. Enhancement of Tm Binding to F-actin 72

3. Summary 74

4. Salmine Inhibition Continued 74

CHAPTER IV: DELINEATION OF THE MINIMUM INHIBITORY REGION

A. SHORTENING FROM THE N-TERMINAL 78

B. SHORTENING FROM THE C-TERMINAL 80

C. FURTHER SHORTENING FROM THE N-TERMINAL 85

D. SUMMARY 87

Page

CHAPTER V: COMPARISON OF THE INHIBITORY SEQUENCES OF VARIOUS SPECIES OF Tn-I

A. Tn-I ACTIVITIES IN A RABBIT FAST ACTOMYOSIN SYSTEM 92

1. Comparison of Chicken Fast, Rabbit Fast and Rabbit Slow Tn-I 92

2. Comparison of Rabbit Fast and Rabbit Cardiac Tn-I 94

B. TN-I ACTIVITIES IN CARDIAC AND HYBRID ACTOMYOSIN SYSTEMS . . 97

1. Hybrid and Bovine Cardiac Actomyosin Assays 97

2. Hybrid Rabbit Actomyosin Assays 99

a. Peptide inhibition 101

b. Tn-I inhibition 101

C. SUMMARY 104

CHAPTER VI: CODA

A. FURTHER INVESTIGATIONS 110

1. Construction of More Analogs 110

2. Location of the Complementary Binding Site 111

3. Studies on Binding and Conformation 112

B. REFLECTIONS ON THE FREQUENCY OF ACTIVE REGIONS THAT ARE SHORT PEPTIDE SEQUENCES ("LINEAR") 113

1. The Improbability of Discovery of "Linear" Active Regions by Cleavage Methods 114

2. The Improbability of Discovery of "Linear" Active Regions by X-ray Crystallography 115

3. Some Examples of Other "Linear" Active Regions of Proteins 117

BIBLIOGRAPHY 119

LIST OF TABLES

		Page
I	Rabbit muscle protein extinction coefficients and molecular weights	32
II	Sequences of CNBr fragment of rabbit skeletal Tn-I, homologous rabbit cardiac Tn-I, homologous rabbit slow Tn-I region and synthetic peptides	58
III	Progress of the synthesis of peptide I	60
IV	Effect of equivalence and coupling number on peptide yield	62
V	Comparison of peptide I on resin and after purification	67
VI	Purity of peptides as demonstrated by amino acid analysis	
VII	History of peptide XII	69
VIII	Effect of ionic strength and peptide I on Tm binding to F-actin	75
IX	Effect of salmine on F-actin-tropomyosin pellet composition	77
X	Effect of shortening the sequence from the NH_2-terminal	79
XI	Effect of shortening the sequence from the COOH-terminal.	81
XII	Effect of the COOH-terminal charge	84
XIII	Further shortening of the sequence from the NH_2-terminal.	86
XIV	Inhibition by rabbit fast and rabbit cardiac peptide analogs of Tn-I in rabbit fast acto-S-1 (A1)ATPase.	98
XV	Activities of rabbit fast and cardiac analogs in hybrid rabbit acto-S-1 (A1) ATPase assays: Skeletal or cardiac actin, fast S-1 (A1)	102
XVI	Activities of rabbit fast and cardiac analogs in hybrid rabbit actomyosin ATPase assays: Skeletal or cardiac actin, cardiac myosin	103
XVII	Activities of rabbit fast and cardiac Tn-I in hybrid rabbit actomyosin ATPase assays: Skeletal or cardiac actin, skeletal or cardiac myosin	106

LIST OF FIGURES

		Page
1.	Structure of striated muscle	4
2.	Sarcomere structure	5
3.	Sarcomere length and tension development	7
4.	T system and Sarcoplasmic reticulum	8
5.	Thin filament structure	13
6.	Steric versus Allosteric control of contraction	18
7.	Steps in solid phase peptide synthesis	26
8.	DEAE-52 Cellulose purification of rabbit skeletal and cardiac S-1	43
9.	DEAE-Sephadex purification of Tn-C	47
10.	Purification of Tn-I	50
11.	Resolving power of Bio-Rex 70 column	64
12.	Elution profile of purification of peptide XII on Bio-Rex 70	66
13.	Inhibition by rabbit skeletal Tn-I, peptide I, and salmine of actomyosin ATPase	71
14.	Effect of ionic strength on peptide I inhibition of actomyosin ATPase	73
15.	Non-essential amino acids of inhibitory sequence of rabbit fast Tn-I	88
16.	Effect of actin concentration on inhibition	90
17.	Inhibitory region sequence of chicken fast; rabbit fast, slow and cardiac Tn-I	93
18.	Inhibition by rabbit cardiac and fast Tn-I of rabbit skeletal actomyosin ATPase	96
19.	Relative activities of rabbit fast and bovine cardiac Tn-I in rabbit skeletal and bovine cardiac actomyosin ATPase assays	100
20.	Gel electrophoresis of rabbit cardiac, fast Tn-I and fast Tn-C	105

Page

21. Inhibition by rabbit cardiac and fast Tn-I of hybrid rabbit actomyosin ATPase: Skeletal or cardiac actin, skeletal and cardiac myosin 107

22. Relation of CNBr inhibitory fragment sequence to biological function 109

ABBREVIATIONS

A_λ - Absorbance at the given wavelength λ

Actomyosin ATPase - the actin activated Mg^{+2} dependent hydrolysis of ATP by myosin

Acto-S-1 (A1) - complex formed between actin and myosin subfragment, containing alkali light chain 1

ADP - Adenosine diphosphate

Ala - Alanine

AMP - Adenosine monophosphate

ATP - Adenosine triphosphate

ANSA - 4-amino-3-hydroxyl-1-napthelenesulfonic acid

Arg - Arginine

Asn - Asparagine

Asp - Aspartic acid

Boc - Tert-butyloxycarbonyl

CD - Circular dichroism

CIDNP - Chemically induced dynamic nuclear polarization

2-ClZ - 2-chlorobenzyl

CNBr - Cyanogen bromide

C-terminal - α-carboxyl terminal

DCC - Dicyclohexylcarbodiimide

DCU - Dicyclohexylurea

DEAE - Diethylaminoethyl

d^3H_2O - Doubly deionized and distilled water

DMF - Dimethylformamide

DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT - Dithiothreitol

$E^{1\%}_{280\ cm^{-1}}$ - Absorbance of a 1% protein solution in a cell with a 1 cm path-length at a wavelength of 280 nm

EDTA - Ethylenediamine-tetra-acetic acid disodium salt

EGTA - Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid

F-actin - Fibrous polymer of G-actin

g - Grams

x g - Times gravity

G-actin - Globular monomers of actin

Gln - Glutamine

Gly - Glycine

HF - Hydrofluoric acid (anhydrous)

Hse - Homoserine

I_{50} - Amount of inhibitor required to achieve 50% of the maximum inhibition

Leu - Leucine

Lys - Lysine

[m] - Concentration expressed as molarity

m.w. - Molecular weight

nm - Nanometers

NMR - Nuclear magnetic resonance

N-terminal - α-amino terminal

Phe - Phenylalanine

PMSF - Phenylmethylsulfonyl fluoride

Pro - Proline

TFA	-	Trifluoroacetic acid
Thr	-	Threonine
Tm	-	Tropomyosin
Tn	-	Troponin
Tn-C	-	Ca^{+2} binding subunit of troponin
Tn-I	-	Inhibitory subunit of troponin
Tn-T	-	Tropomyosin binding subunit of troponin
Tos	-	4-toluenesulfonyl
S-1 (A1)	-	Myosin subfragment 1 alkali light chain 1
S-1 (A2)	-	Myosin subfragment 1 alkali light chain 2
SDS	-	Sodium dodecyl sulfate
Ser	-	Serine
Val	-	Valine

CHAPTER I

INTRODUCTION

A. MUSCLE

> "Like most children the Biochemist, when he finds a toy, usually pulls it to pieces, and he can seldom keep his promise to put it together again. The loveliest toy ever provided by nature for the biochemist is the contractile muscle fibril." [1]

The loveliness of the contractile muscle fibril results from three main factors. Firstly this is a system for transduction. Chemical energy is converted directly to mechanical energy with an efficiency under favorable conditions that approaches 60% (Bull, 1964). Energy conversion of this efficiency using only, carbon, hydrogen, oxygen, nitrogen, sulfur and phosphorus based materials while common in the biological world is unheard of in the man made world.

Secondly, movement has always been closely associated with life itself and while muscle contraction is interesting it has provided clues to such processes as; the movement of chromosomes during mitosis (Nicklas, 1975), the cleavage of a cell during mitosis by the contractile ring (Schroeder, 1975) and the beating of the flagella of a sperm on its long trek to fulfilment (Gibbons, 1975). These three examples only coincidentally emphasize the importance of movement in sex.

Thirdly, the elucidation of how muscle works is almost uniquely accessible to a whole series of investigations. Clues to its function can be found from its ultrastructure using light and electron microscopy and X-ray analysis of paracrystals. The major proteins involved

[1] Albert Szent-Gyorgi "The Chemistry of Muscular Contraction," (Academic Press Inc. New York, N.Y., 1947), p. 1.

can be extracted and the full range of standard biochemical procedures, amino acid analysis, NMR, assaying of enzymatic activity, CD, ultracentrifugation and others can be brought to bear on the problem. In addition to being accessible to so many techniques it is obvious that the ultimate understanding of muscle will involve information about metabolic, control and electrical processes as well as its mere structural arrangement.

The following introduction to muscle will proceed from a gross morphological level to a finer molecular level so that our research can be placed in its proper perspective. Excellent reviews of the material covered are included in Taylor (1979), Mannherz and Goody (1976), Squire (1975), Weber and Murray (1973), Adelstein and Eisenberg (1980) and McCubbin and Kay (1980).

1. Ultrastructure

> "To see actomyosin contract was one of the greatest impressions of my scientific career. Motion is one of the most basic biological phenomena and has always been looked upon as the index of life. Now we could produce it in vitro with the constituents of the cell."[2]

There are at least three kinds of muscle. First is striated or skeletal (voluntary) muscle. The second is cardiac muscle which resembles skeletal muscle except the fibers are branched. The third kind is smooth muscle as exemplified by that of the gastrointestinal tract and the uterus. We will be concerned with only the first two and primarily with striated muscle.

Striated muscle contains many individual muscle fibers or cells. A single nerve controls 100 to 150 muscle fibers (called a motor unit) in an all or none fashion. Graded response is achieved by activating a fraction of the total number of fibers present in the muscle. While

[2] Ibid. pp. 1-2.

skeletal muscle fibers are multinucleated, cardiac fibers have a single centrally located nucleus. The muscle fibers are enclosed by a plasma membrane called a sarcolemma and the majority of the cell is occupied by myofibrils (Fig. 1).

a. Sarcoplasm

The myofibrils are immersed in the sarcoplasm. The sarcoplasm contains glycogen, glycolytic enzymes and intermediates, ATP, ADP, AMP, phosphate, phospho-creatine, creatine and salts. The principal ion is potassium with only small quantities of sodium, calcium chloride and magnesium ions usually being present. Mitochondria and the oxygen transporter myoglobin are also present in the sarcoplasm. These are largely responsible for the red appearance of some muscle fibers. These fibers are slower but undergo fatique less rapidly than their white counterparts. For this reason extensor (antigravity) muscles are primarily composed of red fibers whereas flexor muscles are primarily composed of white fibers.

b. Sarcomere and the sliding filament

Each myofibril is composed of groups of myofilaments which under the phase contrast microscope reveal the characteristic alternating light and dark bands which give striated (striped) muscle its name (Fig. 1c, 2a). The light bands are isotropic, their physical properties are uniform regardless of the direction the measurement is taken, and are called I bands. They are about 1.0 μm long and are transected by a dense line .05 μm thick called the Z line. The dark bands are anisotropic, possess double refraction which is generally exhibited when asymmetric molecules are oriented in a specific direction, and are called A bands. They are about 1.6 μm long when the muscle is at rest and are transected by the dense M line (Fig. 2a). One repeating unit, from Z line to Z line,

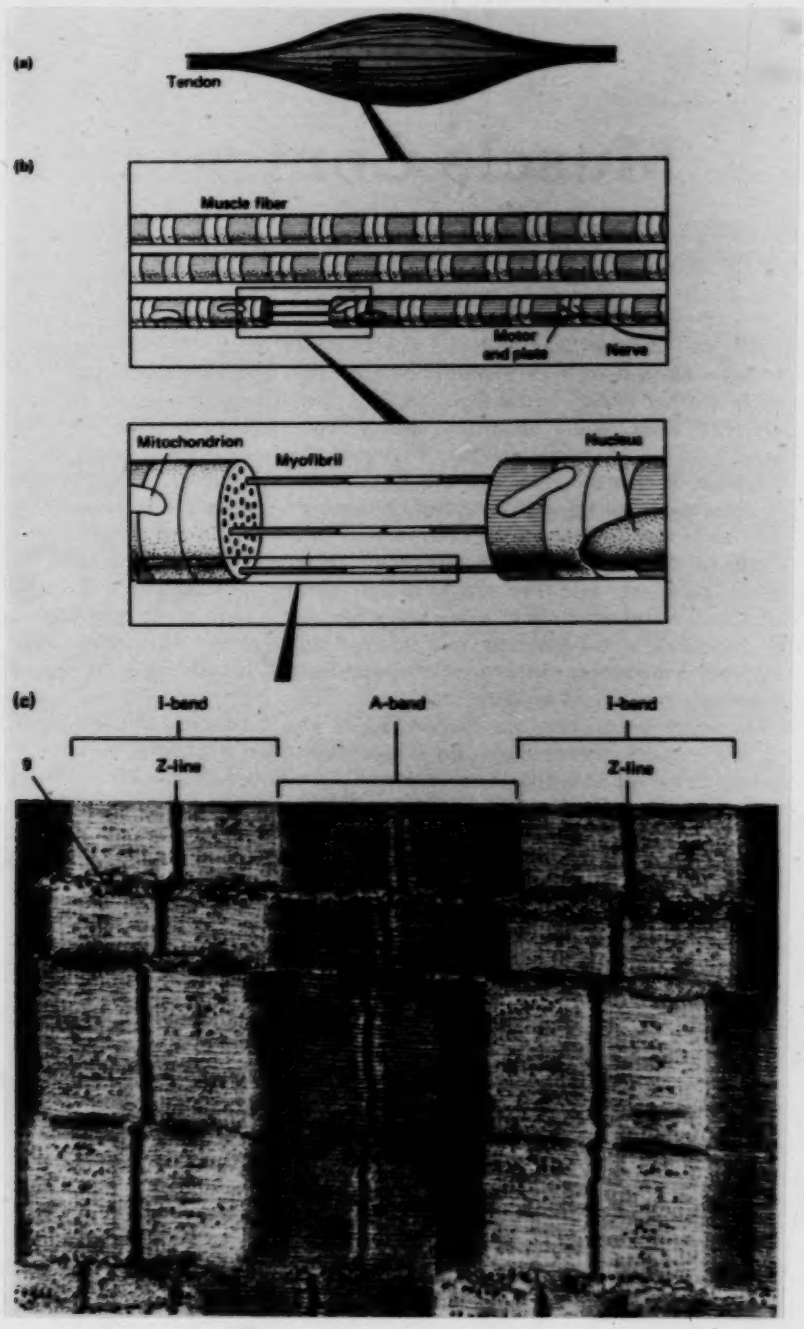

Fig. 1. Structure of striated muscle.
(a) Sketch of a muscle.
(b) Enlargement of muscle showing individual fibers or cells.
(c) Electron micrograph of a portion of a frog sartorius muscle. g indicates dark glycogen granules. [From Bronk, 1973]

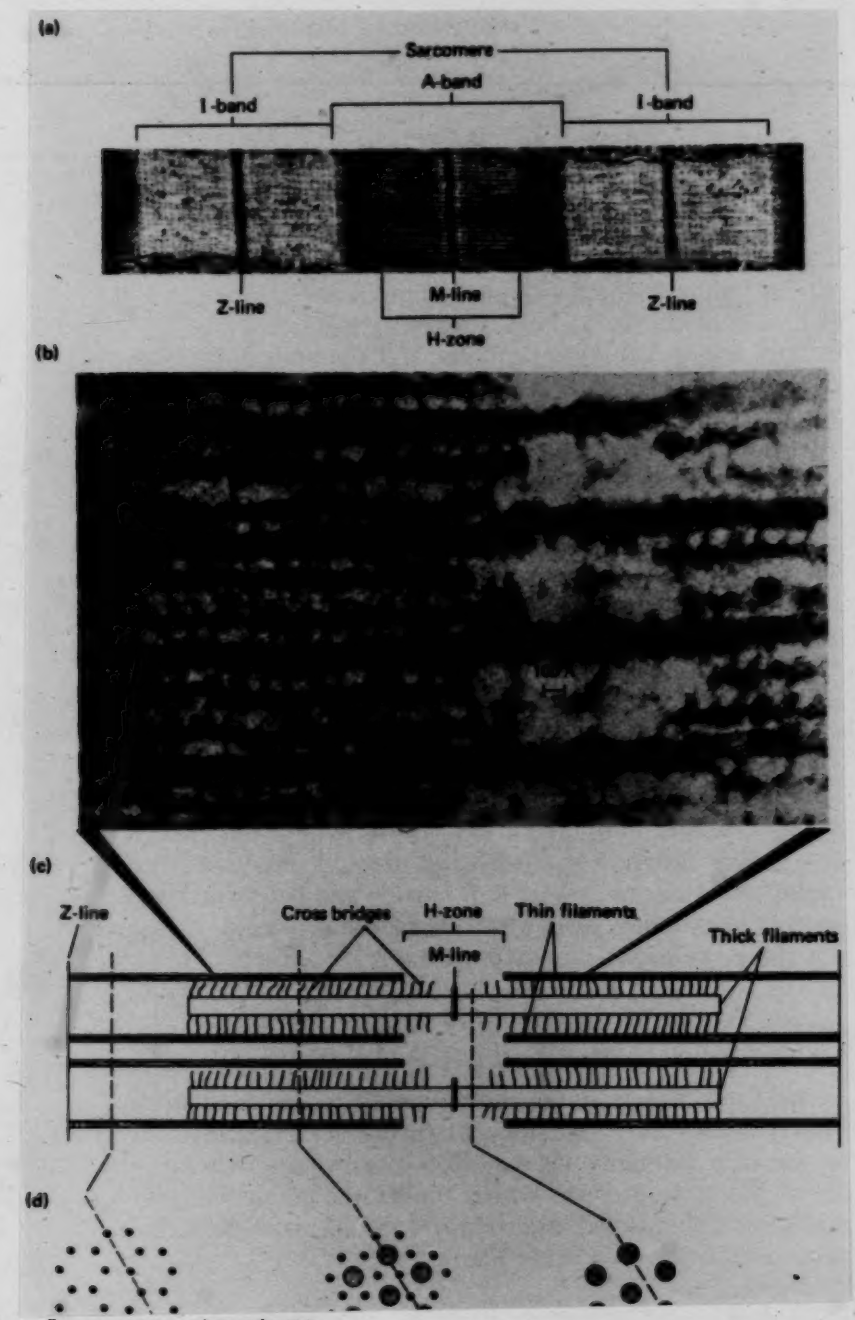

Fig. 2. Sarcomere structure.
(a) Electron micrograph showing A-bands, I-bands, Z-lines, H-zone and M-line.
(b) High resolution electron micrograph showing detailed structure of thick and thin filaments. Note especially the cross-bridges linking the thick and thin filaments.
(c) Schematic of sarcomere shown in (b).
(d) Cross sections of sarcomere shown in (c). [From Bronk, 1973]

is about 2.5 μm long and is called a sarcomere.

Electron micrographs of the A band (Fig. 2b) have shown it to be composed of both thick and thin filaments, 16 nm and 6 nm in diameter respectively. These also reveal connections or crossbridges between the two kinds of filaments. The thick filaments are present throughout the entire A band whereas the thin filaments begin at the Z line and extend only partially into the A band (Fig. 2c and d).

Observation of sarcomere length during contraction has revealed that the A bands always remain constant in length while the I bands can virtually disappear in a strong contraction. This led to the sliding filament theory (Huxley and Hanson, 1954 and Huxley and Niedergerke, 1954) which maintains that contraction occurs by a sliding of thick and thin filaments past one another. This shortening may occur to such an extent that thin filaments slide past one another or the thick filament (A band) shortens slightly. Both of these result in a reduction of tension (Fig. 3; points 4,5,6). Thus tension increases with increasing overlap of thick and thin filaments until the thin filaments start to slide past one another, then the tension decreases (Fig. 3).

c. Sarcolemma, Transverse tubules and Sarcoplasmic reticulum

Finally the outer membrane, the sarcolemma, mentioned above is continuous with a system of transverse tubules (T system) that penetrate muscle fibers near the Z lines in some muscles and the A-I junction in others. When the sarcolemma becomes depolarized due to the action of a nerve impulse this depolarization continues along the transverse tubules to all the sarcomeres in the fiber (Fig. 4). Very close but not connecting to the T system is the highly specialized endoplasmic reticulum of muscle cells which is called the sarcoplasmic reticulum. When the T

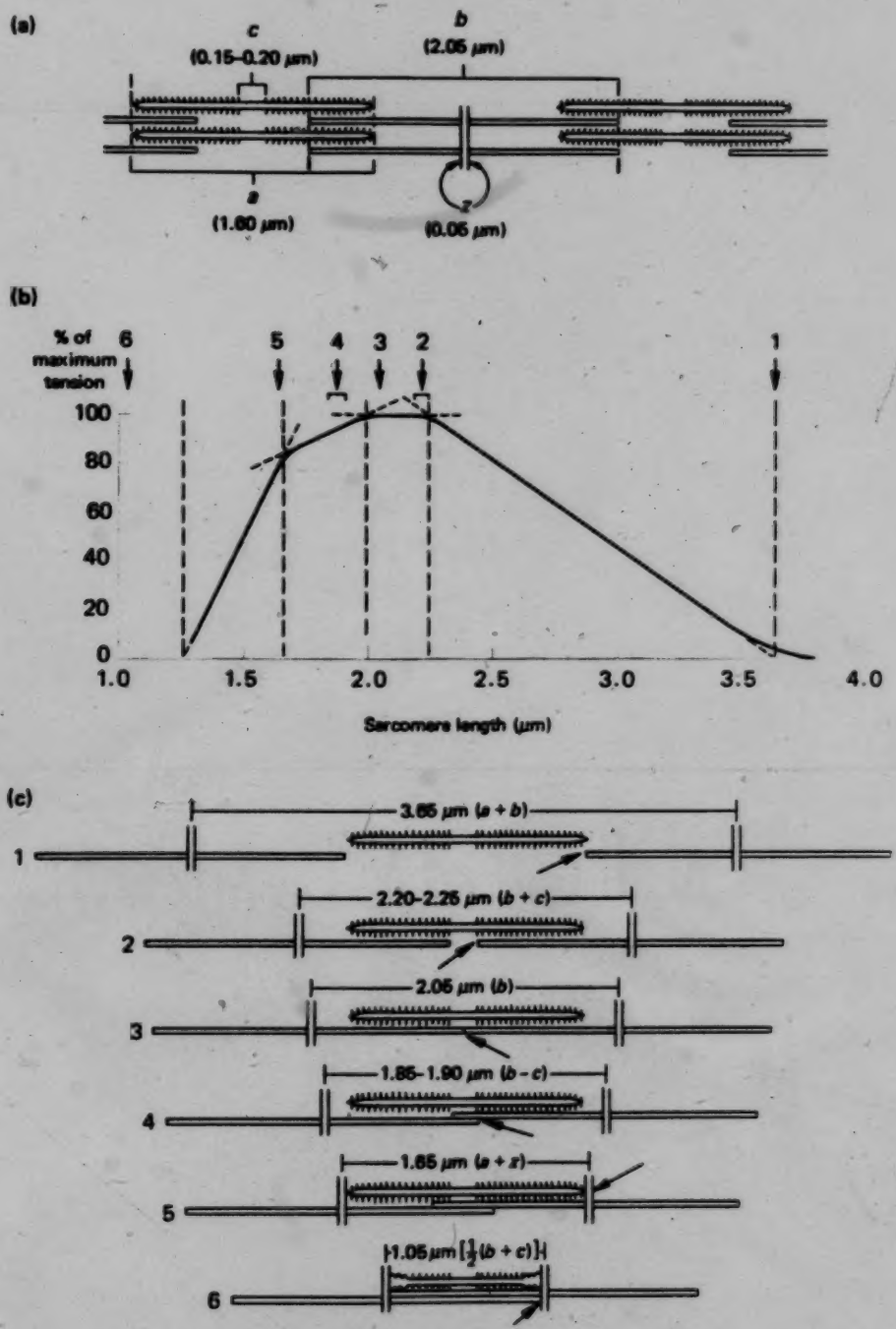

Fig. 3. Sarcomere length and tension development.
(a) Dimensions of thick and thin filaments and regions.
(b) Tension development versus distance between adjacent Z-lines. Numbers above curve refer to (c).
(c) Diagrams showing degree of overlap. [From Gordon et al., 1966]

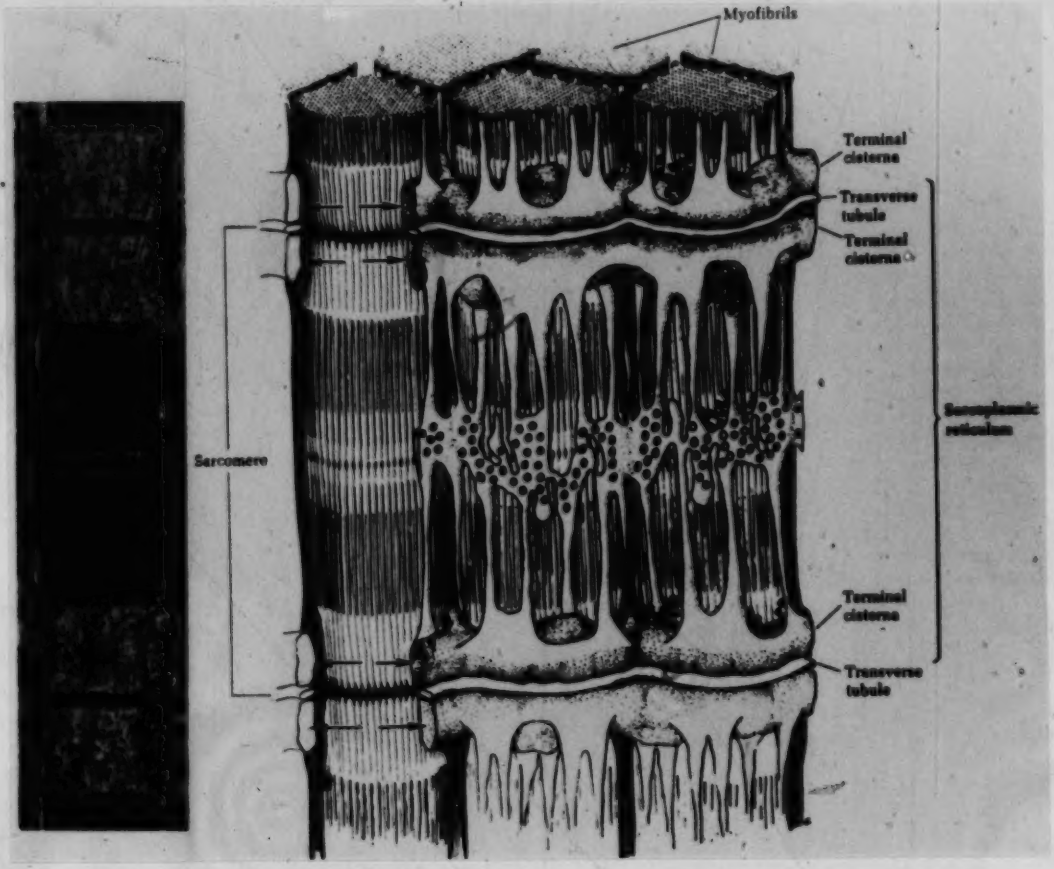

Fig. 4. T-system and Sarcoplasmic reticulum. The electron micrograph is of frog sartorius muscle. The drawing shows the vertical bundles which are individual myofibrils and the positions of transverse tubules and sarcoplasmic reticulum. [Adapted from Peachey, 1965]

system depolarizes, the closely associated sarcoplasmic reticulum becomes permeable, and Ca^{2+} ions escape from the terminal cisternae or sacs of the sarcoplasmic reticulum to a concentration greater than 10 μM. That Ca^{2+} is the actual signal for sarcomere contraction was first demonstrated by the injection of Ca^{2+} by micropipette into muscle which resulted in localized contraction (Heilbrum and Wiercinski, 1947). After the nerve impulses and hence the depolarization of the sarcolemma and T system have stopped, the membrane of the sarcoplasmic reticulum is no longer permeable and Ca^{2+} is pumped in an ATP requiring process into the terminal cisternae. It might be noted that many slow fibers or small muscles have poorly developed sarcoplasmic reticulums and few if any transverse tubules. In these cases the release and uptake of Ca^{2+} may depend on the mitochondria or sarcolemma. While slow, this process may be sufficient for small or slow fibers. After Ca^{2+} has been reduced to a concentration below .2 μM the myofibrils are then stretched out again by the action of antagonistic muscles; extensor muscles, if those that contracted were flexors, and vice versa.

2. Infrastructure

In the next sections the thin and thick filament will be described on a molecular level.

a. Thick filament

Muscle can be extracted with a solution containing 0.6 M KCl and when this KCl concentration is reduced a precipitate consisting primarily of one protein results. Examination of muscle treated in this fashion reveals that the A band has reduced density. This protein, called myosin, accounts for 54% of all the protein of a myofibril (Hanson and Huxley, 1957).

Myosin, a highly asymmetric protein, has a molecular weight of 460,000. It consists of 6 polypeptide chains; in skeletal muscle these are 2 heavy chains m.w. 200,000, 2 light chains m.w. 18,000 and 2 non-identical light chains m.w. 16,500 and 21,000. The major portion of the carboxyl end of the two heavy chains is α-helical and are wound around one another in a coiled-coil fashion. The light chains are complexed with the globular amino terminal portion of the heavy chains. The two identical light chains can be removed by treatment with DTNB while the non-identical ones can be removed by harsh treatment with alkali. Myosin can be made to aggregate into structures closely resembling thick filaments by lowering the ionic strength. The molecules arrange themselves in a tail-to-tail bipolar fashion such that the coiled-coil portion is bound but the globular light chain containing region is free to interact. If the aggregation occurs in the presence of EDTA, to chelate divalent cations especially Ca^{2+}, and then digested with chymotrypsin, three major fragments occur. Two are the globular portions, each containing one of the so called alkali light chains. These are designated S-1 (A1) and S-1 (A2) and possess a portion of the heavy chain with m.w. of 90,000 and the 21,000 or 16,500 m.w. light chains respectively. The DTNB chains are destroyed by the digestion. The third fragment is the rod-region and has a m.w. per polypeptide of 117,000 (Weeds and Pope, 1977).

Myosin possesses ATPase activity which has pH optima at 6.0 and 9.5, is inhibited by Mg^{2+} and exhibits optimal activity only in solutions of high ionic strength, e.g., 0.5 M KCl. S-1 possesses the same activities and it appears that the DTNB light chains are not essential for activity. The myosin Mg^{2+} ATPase activity can be greatly activated

by the addition of actin. This actin activated ATPase in contrast requires Mg^{2+} and occurs at physiological ionic strength. Unfortunately at this ionic strength myosin is marginally soluble. As a consequence S-1, which is soluble under these conditions, is often used in place of myosin. Both myosin and S-1 actin activated ATPase activities are sensitive to KCl concentration with the activity decreasing as KCl concentration increases. This occurs because the apparent binding constant of myosin heads to actin decreases (Eisenberg and Moos, 1968; Wagner et al., 1979). These actin activated myosin ATPase activities are so low at physiological ionic strength ($[Na^+] + [K^+] + [Cl^-]$, 0.10 - 0.18 M; Harper, 1965) that assays are usually carried out at much lower ionic strength (0-.03 M KCl).

b. Thin filament

i. Actin

Actin is the major component of the thin filaments and represents 20 to 25% of all myofibrillar protein (Hanson and Huxley, 1955). This protein can occur as G-actin (globular monomer) or F-actin (fibrous polymer of G-actin). Its molecular weight including one bound ATP molecule is 42,000. On polymerization, usually triggered by increasing the ionic strength of a solution of G-actin to 2 mM Mg^{2+} and 50 mM KCl, the ATP is hydrolyzed. F-actin therefore contains ADP which along with a tightly bound divalent cation is essential for stabilizing the actin. It has been suggested that the function of the salt in polymerization is to reduce the repulsion of G-actin monomers and allow intermolecular attractions to dominate. Structurally F-actin is composed of two strands of G-actin monomers coiling around one another with a helix diameter of 7-8 nm and a repeat of 36 nm which contains about 13 monomers (Moore

et al., 1970, Fig. 5).

ii. Tropomyosin

> "A new fibrous protein called 'tropomyosin' has been described recently. Its quantity is rather small as compared to that of actin or myosin and since there is no evidence that it is involved in the contractile mechanism, its discussion will be omitted." [3]

Tropomyosin accounts for 12-15% of myofibrillar protein. It is composed of two α-helical polypeptides wrapped around one another in a coiled-coil fashion with a total molecular weight of 66,000 and a length of 40 nm. Tropomyosin molecules are linked in a head to tail fashion in the valley formed by the coiled F-actin filaments. This results in a 7 to 1 molar ratio of actin monomers to tropomyosin monomers in the thin filament (Potter and Gergely, 1974). Rabbit skeletal muscle contains two major forms of the polypeptide chain which are present in tropomyosin. These chains have been designated α and β and were found a 3 or 4:1 ratio in rabbit fast skeletal muscle. In contrast rabbit cardiac tropomyosin contains only one kind of chain and this has been shown to be identical in sequence to rabbit skeletal α-tropomyosin (Lewis and Smillie, 1980).

iii. Troponin

Troponin composed of three polypeptide chains, has a m.w. of 76,000 and in conjunction with tropomyosin regulates contraction in vertebrate skeletal muscle. Troponin is present in a one to one ratio with tropomyosin (Potter and Gergely, 1974). Its three subunits are: Tn-T (binds tropomyosin) m.w. 30,503 (Pearlstone et al., 1977), Tn-I (inhibits actomyosin ATPase) m.w. 20,900 (Wilkinson and Grand, 1975a) and Tn-C (binds Ca^{2+}) m.w. 18,000 (Collins et al., 1973). Tn-I and Tn-T are very basic with isoelectric points around 9.3 (Wilkinson, 1974)

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[3] Ibid. p. 2.

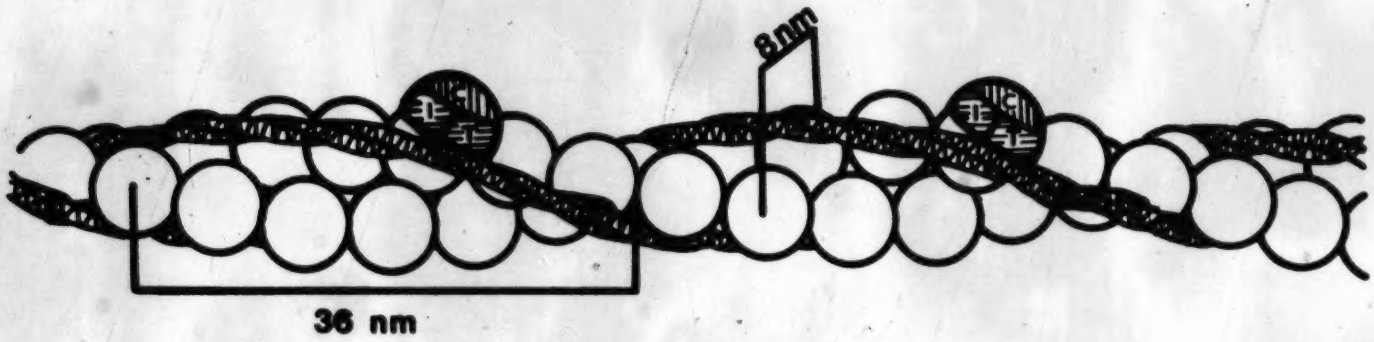


Fig. 5. Thin filament structure. Open circles represent actin monomers. Long α-helical molecules represent tropomyosin. Oval shaded molecule represents the troponin complex containing Tn-I, Tn-C and Tn-T. The diagram summarizes the probable interactions of these including Tn-I to actin. [Adapted from Cohen, 1975]

and 8.8 (Hartshorne and Dreizen, 1972) respectively. The isoelectric point of Tn-C is around 4.1 (Hartshorne and Dreizen, 1972) and thus very acidic.

iv. Thin filament protein interactions

Tropomyosin binds to F-actin in a manner dependent on the ionic strength of the solution and/or the presence of Tn-I (Eaton et al., 1975). In solutions containing 1 mM Mg^{2+} and 2 mM ATP, where the concentration of Mg^{2+} not bound to ATP must be very low, concentrations of KCl 30 mM or less, resulted in very little binding of Tm by F-actin. At a concentration of 100 mM KCl about half the maximal binding of Tm to F-actin was observed. When the Mg^{2+} concentration was 5 mM and the ATP concentration 2 mM resulting in a concentration of free Mg^{2+} of approximately 3 mM, maximal binding was observed at 30 mM KCl. The binding of Tm correlated with increasing inhibition of the actin activated myosin ATPase (Eaton et al., 1975) and F-actin rigidity (Oosawa et al., 1972).

Although Tn-I does not bind to Tm at 0.2 M KCl (Potter and Gergely, 1974; Hitchcock, 1975), Tn-I increases the F-actin affinity for Tm (Hitchcock, 1975). At ionic strengths that resulted in less than optimal binding of Tm by F-actin, the addition of Tn-I increased the binding of Tm (Eaton et al., 1975). Taken together these results suggest either that tropomyosin alters the affinity of actin for Tn-I and vice-versa, or that Tn-I binding occurs at a site made of actin and tropomyosin. When tropomyosin was optimally bound the maximum inhibition observed was 60% and the addition of Tn-I increased the inhibition to greater than 80% without increasing the amount of bound tropomyosin (Eaton et al., 1975). In summary, depending on the concentrations of mono and divalent cations, these effects may occur. Tropomyosin may

bind to F-actin and inhibit the actin activated myosin ATPase; or Tn-I may increase the binding of Tm to F-actin with the resultant inhibition; or after Tm has been maximally bound Tn-I may exert an additional inhibition of its own.

Tn-C and Tn-I interact and this complex does not inhibit the actomyosin ATPase regardless of whether Ca^{2+} or tropomyosin are present or absent. In contrast while the Tn-C-I complex does not bind to F-actin or to F-actin-Tm in 50 mM NaCl and the presence of Ca^{2+} (0.1 mM), the complex will bind to F-actin-Tm in the absence of Ca^{2+} (Hitchcock et al., 1973). Since this results in no inhibition the Tn-C-I complex may bind to F-actin-Tm in a manner that does not inhibit. Also Tn-C and Tn-I bind strongly in the presence of 6 M urea and 10^{-3} M Ca^{2+} but are dissociated in 6 M urea and 0.1 mM EGTA (Perry et al., 1972). Thus the complex is affected by Ca^{2+} even if it does not dissociate under physiological conditions as evidenced by the above inhibition and binding studies.

Tn-C and Tn-T interact in a manner that is strengthened by Ca^{2+} (McCubbin et al., 1974); Mani et al., 1974; Greaser and Gergely, 1973) and Tn-T, of course, binds to tropomyosin or to F-actin-Tm independently of Ca^{2+} (Potter and Gergely, 1974; Hitchcock, 1975).

Difficulty arises in studying interactions of Tn-I and Tn-T at physiological conditions as Tn-I is insoluble below an ionic strength of 0.2 M KCl and Tn-T below 0.3 M KCl. For this reason SDS polyacrylamide gel electrophoresis at low ionic strength showed no interaction of Tn-I and T (van Eerd and Kawasaki, 1973). However the following evidence indicates that they do interact. In troponin chemical and photochemical crosslinkers indicate that two subunits are within 0.6 nm or

touching one another (Hitchcock, 1975a; Sutoh and Matsuzaki, 1980). Spectral changes were observed when the two proteins were properly reduced and allowed to interact (Horowitz, 1979; Hincke et al., 1979). When the two proteins were mixed at salt concentrations of 0.3 M to 0.5 M NaCl gel filtration gave only one peak (Horwitz, 1979; Hincke et al., 1979). Fragments of Tn-T were found to interact with Tn-I by affinity chromotography and gel filtration (Katayama, 1979, Pearlstone and Smillie, 1980).

v. Two site model

> "Ah! that's a very, very interesting thing! .... Because Jacques (Monod) has since told me that a dogma is something which a true believer cannot doubt!" Crick laughed. "But that wasn't what was in my mind. My mind was that a dogma was an idea for which there was no reasonable evidence." [4]

The interactions mentioned in the previous section and the combined X-ray diffraction studies and electron microscopy studies of muscle (Huxley, 1972; Haselgrove, 1972; Parry and Squire, 1973; Wakabyashi et al., 1975) have led to the two site model for control of muscle contraction (Hitchcock et al., 1973; van Eerd and Kawasaki, 1973; Potter and Gergely, 1974). This model states that in the presence or absence of Ca^{2+} the troponin complex is always anchored to F-actin-Tm via Tn-T. In the absence of Ca^{2+}, Tn-I is bound either to actin or to a site jointly made up of actin and tropomyosin. In the presence of Ca^{2+}, the Tn-I F-actin-Tm link is broken and myosin and actin are free to interact. The tropomyosin serves to amplify these signals by carrying it to all 7 actins.

[4]Horace Freeland Judson "The Eighth Day of Creation" Simon and Schuster. New York, N.Y., (1979) p. 337.

The previously mentioned X-ray diffraction and electron microscopy studies have established that when muscle contracts (Ca^{2+} present), tropomyosin is found closer to the center of the groove of the F-actin double helix than when the muscle relaxes. As a result of this observation two possibilities arise. Tropomyosin which is not bound to F-actin through troponin I may have one of two thermodynamically favored positions. If the position closer to the central groove of F-actin was the more stable position the addition of Ca^{2+} and resultant loss of F-actin-Tn-I binding would result in the spontaneous movement of Tm to the close position. If the far position was the most stable the addition of Ca^{2+} would release the binding of F-actin by Tn-I. Then a conformational change would be induced in Tm or the Tm and F-actin to cause the Tm to move to the otherwise unfavorable close position.

The X-ray and electron microscopic studies cited above do not indicate whether Tm and Tn are located on the same side of the actin as reacts with the myosin or the opposite. A recent study (Seymour and O'Brien, 1980) indicates that Tm and Tn are located on the opposite side of the actin from its myosin interaction site. If this is correct the simple model which suggests Tm and Tn sterically block the myosin interaction site cannot be true. The steric model and allosteric model are represented in Fig. 6a and 6b respectively.

Fig. 6b represents one conception of how an alternative to the steric model might look. The most compelling evidence against the steric model (Chalovich et al., 1981; Greene and Eisenberg, 1980) suggests a slightly different picture. These studies have shown that F-actin-Tm-Tn bound S-1·ATP or S-1·ADP·Pi to nearly the same extent whether Ca^{2+} was present or not. In fact the binding of S-1·ADP or S-1 were the

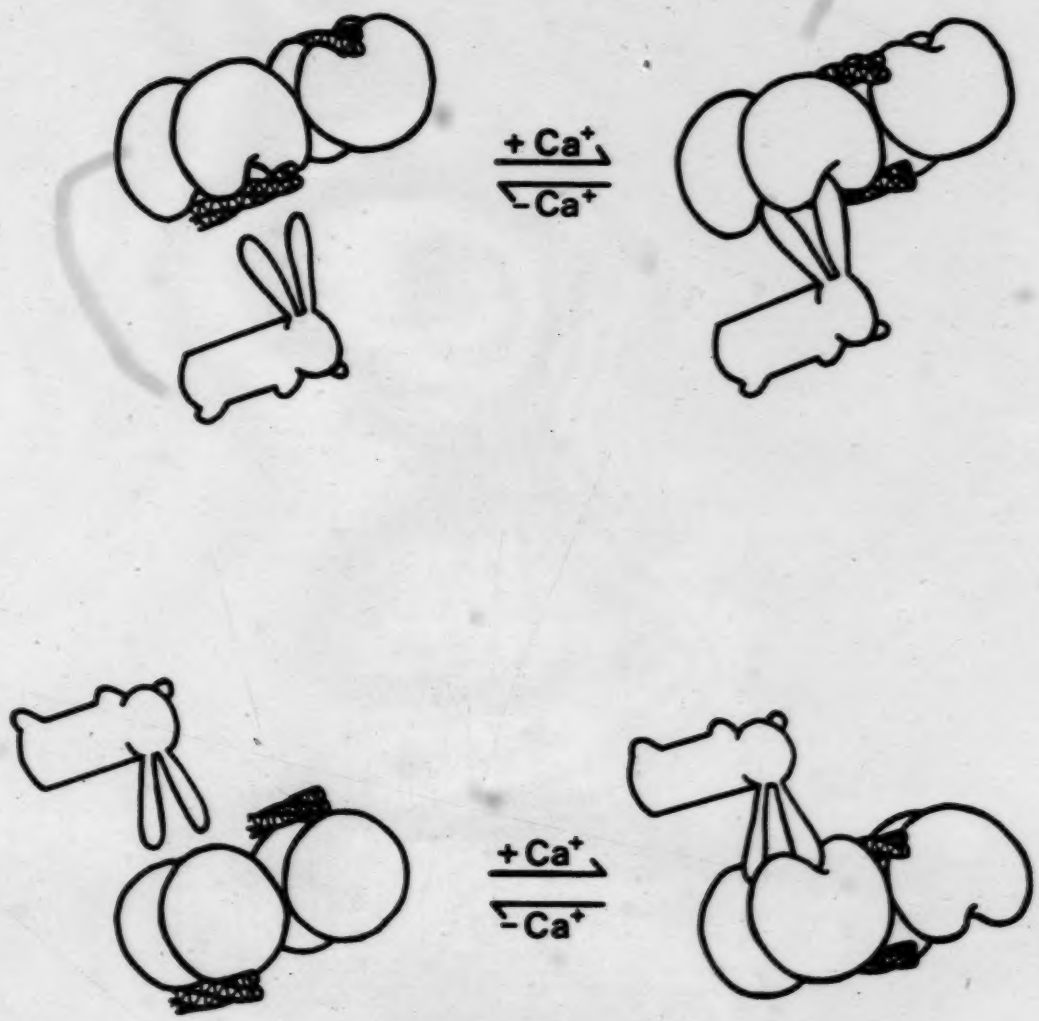


Fig. 6. Steric versus Allosteric control of contraction.

(a) Steric blocking of actin-myosin interaction. Tropomyosin physically blocks the interaction of myosin until Ca^{2+} combines with troponin and thus must be on same side of actin as the myosin combining site.

(b) Allosteric blocking of actin-myosin interaction. Actin-myosin interaction site created as tropomyosin moves in response to Ca^{2+} binding to troponin. Tropomyosin may be on opposite side of actin from myosin combining site. Open circles, actin; long helical molecule, tropomyosin; molecule with ears, myosin section.

only species whose binding was reduced in the absence of Ca^{2+}. These results suggest that the primary effect of Tm-Tn in the absence of Ca^{2+} was not to sterically block the binding of S-1·ATP or S-1·ADP·Pi. Its effect was to destabilize the binding of S-1·ADP or S-1 and possibly to decrease the transition rate of acto-S-1·ADP·Pi to acto-S-1·ADP. If these results are correct then a certain fraction of myosin cross bridges remain attached to F-actin even in relaxed muscle. This is compatible with the lack of stiffness in relaxed muscle only if the attached cross bridges are weakly attached and in rapid equilibrium with unattached bridges.

vi. Lin Yu-tang and the thin filament

Although the two site model is often taken as dogma there is considerable evidence that it is not completely descriptive of the way troponin, tropomyosin and actin fulfil their roles in the regulation of contraction and relaxation. Firstly, two of the more important papers for the two site model (Hitchcock et al., 1975; Potter and Gergely, 1974) contain data difficult to rationalize with the model. For instance both report substantial binding of Tn to F-actin and that this binding is unaffected by the presence and absence of Ca^{2+}. Since the only possible interaction site is Tn-I and it is supposedly Ca^{2+} sensitive does this binding represent "non-specific" binding or overlooked binding? Furthermore, while the binding was not Ca^{2+} sensitive, the inhibition the complex caused was (Hitchcock et al., 1975). This suggests that the interaction may be specific and that Tn-I may be able bind without inhibiting. This was also mentioned as a possibility for the Tn-I-Tn-C complex (Hitchcock et al., 1973) which binds F-actin-Tm in the absence of Ca^{2+} but does not inhibit the actomyosin ATPase.

Therefore the actual physical dissociation of Tn-I from actin may not be necessary for the release of inhibition. Another possibility in explaining why Tn binds to F-actin in the presence or absence of Ca^{2+} is implied by the finding that 0.15 mol of Tn-T/7 mol of actin occurs in 0.4 M KCl (Potter and Gergely, 1974). Thus it is possible at physiological conditions that Tn-T binds to F-actin and this in Tn would result in a Ca^{2+} insensitive link that would occur in the absence of Tm.

Secondly, the Tn-T, Tn-I interaction was not suspected when the two site model was drafted. As a result the reconstitution of activity with the addition of Tn-T to Tn-I - Tn-C complex was interpreted as a result of the second (and only known) interaction, the Tn-T-Tm link. It is possible that Tn-T interacts with Tn-I in a manner that allows it to bind and exhibit Ca^{2+} sensitive inhibition. This possibility is intriguing in light of the evidence that Tn-T and Tn-I together can inhibit actomyosin ATPase as well as Tn-I and Tm (Eisenberg and Kielley, 1974). This may mean that Tn-T binds to actin in a manner similar to Tm or that the Tn-T changes the Tn-I conformation directly.

Thirdly, while the model proposes that Tn-I of an actin-Tm-Tn complex does not bind directly to F-actin in the presence of Ca^{2+}, evidence exists that Tn-I is close enough to crosslink to actin. The crosslinking of Tn-I to F-actin occurs in the presence or absence of Ca^{2+} although less crosslinks in the presence of Ca^{2+} (Sutoh, 1980). This leaves open the possibility that Tn-I binds (although possibly in a different manner) to actin whether Ca^{2+} is present or not.

One final comment on the potential dangers of extrapolating from studies of components to the behaviour of components in a system is in order. It is worth noting that other crosslinking studies of whole Tn

show that its crosslinking pattern is insensitive to the presence or absence of Ca^{2+} but Tn combined with F-actin-Tm manifests a different crosslinking pattern in the presence of Ca^{2+} compared to that found in its absence (Sutoh and Matsuzaki, 1980).

Lastly, the model concentrates on regulation by troponin and tropomyosin yet it has been shown (Poo and Hartshorne, 1976) that actin crosslinked with glutaraldehyde can activate the actomyosin ATPase activity and bind Tm and Tn but cannot be turned off. Therefore actin is probably not just a passive part of the regulatory mechanism.

Although many of these observations may turn out to be wrong or misinterpretations it seems like a good idea to listen to wisdom translated by Lin Yu-tang.

> "Those who rely upon the arc, the line, the compass and the square to make correct forms injure the natural constitution of things...Things in their original nature are curved without the help of arcs, straight without lines, round without compasses and rectangular without squares; they are joined together without glue and held together without cords... For a duck's legs, though short, cannot be lengthened without dismay to the duck, and a crane's legs, though long cannot be shortened without misery to the crane. That which is long in nature must not be cut off, and that which is short in nature must not be lengthened." [5]

c. Rigor complex

Rigor complex is a special case of the interaction between thick and thin filaments. It has been defined as complexes between actin and myosin (or S-1) free of bound nucleotide. In the absence of ATP all actomyosin complexes are rigor complexes; at infinite ATP there are none. The formation of "rigor complexes" is not prevented by troponin and tropomyosin, even in the complete absence of calcium (Bremel and

[5] Lin Yu-tang (in The Tao of Science, R.G.H. Sui, M.I.T. Press Cambridge, Mass.) p. 97.

Weber, 1972). In essence it has been suggested that rigor complexes can cooperatively potentiate the actin filament. Thus the formation of many rigor complexes may result in uncomplexed monomers of actin of the same helix being turned on even if troponin and tropomyosin are present and Ca^{2+} is not (usually the "off" state) (Bremel et al., 1972; Bremel and Weber, 1972; Weber and Murray, 1973). Two factors therefore influence the extent of rigor complex formation. Increasing either the ATP concentration or the actin to myosin ratio can decrease the incidence of rigor complex formation.

3. Short Summary of Events in Muscle Contraction

The sarcolemma of the muscle fiber is depolarized by nerve impulses and this is transmitted through the transverse tubules to the interior of the muscle. The sarcoplasmic reticulum cisternae are as a result made permeable to Ca^{2+} and the concentration of Ca^{2+} rises above 10^{-6} in the myofibril. Tn-C on the thin filament binds Ca^{2+} undergoing a conformational change which is transmitted via Tn-I, Tn-T and Tm resulting in actin now being accessible to myosin. The myosin which is bathed in Mg ATP now interacts through its crossbridges in a cyclical manner with actin. Each myosin contains two tightly bound molecules of ADP and phosphate in a high energy state. This myosin interacts with an actin monomer (in the thin filament) and undergoes a conformational change that changes the crossbridges angular relationship to the thick filament, resulting in movement of the thick filament along the thin filament, and the unbinding of the ADP and phosphate. The actin-myosin rigor complex is dissociated by the binding of Mg ATP to the myosin and this is hydrolyzed to ADP and phosphate to give a high energy state completing the cycle. The contraction and hence the cycle continue as

long as nerve impulses arrive and maintain the depolarization. The Z lines move closer together but the A band does not shorten. ATP is regenerated from ADP and creatine phosphate as well as by other metabolic processes. When the nerve impulses cease the sarcoplasmic reticulum takes up Ca^{2+} and the Ca^{2+} concentration is brought below 10^{-6}M. With the loss of Ca^{2+} the Tn-C undergoes the reverse conformational change and the Tn-Tm complex now prevents any further interaction of actin and myosin. The myofibrils and correspondingly the sarcomeres are now free to be stretched through the action of antagonistic muscles to their original length.

4. Inhibition of Actomyosin ATPase

a. Troponin-I

Tn-I is a highly basic protein whose sequence in many different species and muscle types is known (Wilkinson and Grand, 1978). At neutral pH it has a total positive charge of +9. While Tn-I inhibits actomyosin ATPase it does so at ratios of Tn-I/Actin considerably higher (Syska et al., 1976) than the 1 to 7 ratio of actin filaments (Bremel and Weber, 1972). To achieve inhibition at such a ratio Tm must be present. Tm can also inhibit actomyosin ATPase and this inhibition occurs at ratios greater than physiological (Eaton et al., 1975). This inhibition can be increased by raising the ionic strength. Inhibition by tropomyosin alone reaches a maximum of 60% of the activity of uninhibited actomyosin and has been directly related to the extent of Tm binding to F-actin (Eaton et al., 1975). To achieve inhibition of close to 100% at ratios of protein near to that found physiologically both Tn-I and Tm must be present. Tn-I promotes the binding of Tm to actin and conversely Tm promotes the binding of Tn-I to actin. In both

cases inhibition of ATP hydrolysis is also enhanced. This has led to speculation that either Tn-I binds to a site jointly made up of actin and Tm or an actin site under the conformational direction of Tm and vice versa. Crosslinking studies of Tn-I in troponin (Sutoh and Matsuzaki 1980) show that it binds to Tn-C, Tn-T and actin but not Tm and thus support the latter possibility. Tn-I must be reduced to remove intermolecular aggregates which lower its activity.

b. Basic proteins

It has been reported that many basic proteins, apparently unrelated in any fashion to Tn-I also inhibit actomyosin ATPase (Syska et al., 1976). These inhibitors include salmine and lysozyme. In contrast to Tn-I both these inhibitors demonstrate significantly lower activity in the presence of Tm than in its absence.

c. Tn-I fragments

A 21 residue cyanogen bromide fragment of Tn-I has the ability to inhibit 45-75% as well as Tn-I when compared on a molar basis (Syska et al., 1975). The original sequence reported for this fragment (Wilkinson and Grand, 1975a) contained twenty-two amino acids. After our work was in progress a correction was announced that changed the sequence 111-115 from Leu-Arg-Arg-Arg-Val to Leu-Arg-Arg-Val (Wilkinson and Grand, 1978). This deletion of one arginine was taken into account in the construction of any peptides constructed after this publication appeared. The inhibition of the actomyosin ATPase caused by this cyanogen bromide fragment was greatly decreased in the absence of tropomyosin. The most salient feature of this fragment is the large number of basic residues present, 8 of 21.

B. SOLID PHASE PEPTIDE SYNTHESIS

Several reviews of solid-phase peptide synthesis have been written but by far the most comprehensive is that of Erickson and Merrifield (1976) although the more recent review by Birr (1978) is also good. The best article about laboratory techniques, solvent preparation etc. is Stewart and Young (1969).

Solid-phase peptide synthesis in essence, involves the cylical coupling of activated amino acids and less frequently peptides, to a resin support or the end of a peptide bound to a resin support. The most common direction this kind of synthesis takes is to have the α-carboxyl group attached to the resin and attach subsequent amino acids to the α-amino terminal. Advantages of this approach include the existence of α-amino protecting groups that prevent racemization during coupling, the ability to wash away reagents and decomposition products while retaining the peptide and finally since the reagents are in solution, they can be used at a high enough concentration to drive the reaction to completion. The major disadvantage is that impurities arising from deletion or modification of amino acids of the peptide, remain bound to the resin. Our peptides with a maximum of twenty-two residues were small enough that purification did not appear to be insurmountable and given the advantages of carboxyl to amino terminal assembly this was the route we chose.

The steps in solid-phase synthesis are outlined in Fig. 7. The cesium salt of the first amino acid (with suitable blocking groups) is used to covalently link the amino acid to the resin. The resin most often used is a copolystyrene-chloromethyl resin. This amino acid is then deprotected at its α-amino group by trifluoroacetic acid. The TFA salt is neutralized with the base diisopropylethylamine leaving the

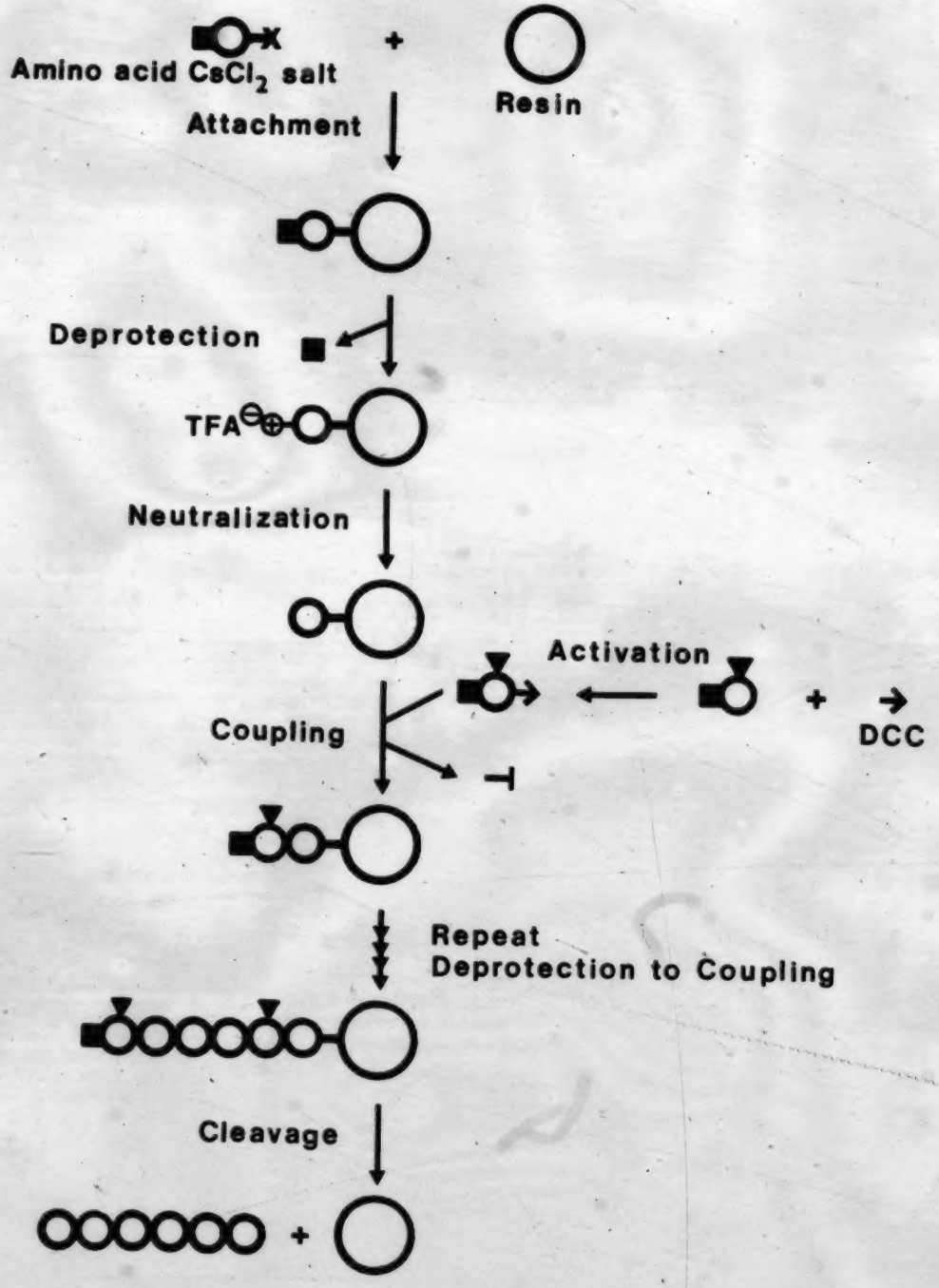


Fig. 7. Steps in solid phase synthesis. Small circles represent amino acids; black squares, N-terminal blocking groups; black triangles, side chain protecting groups stable to deprotection conditions but not cleavage conditions; arrows, DCC activating group; X, Cs atom; ⊣, DCU by-product of coupling reaction.

amino terminal uncharged and free to couple. Coupling occurs by adding another protected amino acid which has its carboxyl group activated with dicyclohexylcarbodiimide, a good leaving group. It is the repetitive nature of this cycle, deprotection, neutralization and coupling, which enables solid phase peptide synthesis to be so easily automated. Any amino acid with a side chain that could undergo a side reaction is blocked with a group that is not susceptible to any of the reagents used in this cycle. When the synthesis is complete the peptide is cleaved from the resin with anhydrous HF which simultaneously removes the side chain protecting groups.

Modifications to this scheme are possible. To construct a peptide that has a carboxyl terminal amide it is only necessary to use a benzhydrylamine resin. Here the first amino acid is attached by a DCC coupling, in place of attachment using the cesium salt of the protected amino acid. On completion of the synthesis HF cleavage of the peptide from the resin generates the C-terminal amide. To obtain a peptide with an N-terminal acetyl group it is only necessary to couple acetic acid with DCC or react acetic anhydride to the N-terminal deprotected and neutralized peptide.

C. PURPOSE OF THIS STUDY

Peptide synthesis has been a very powerful tool for examining certain aspects of the relationship of protein structure to biological function. Such studies have concentrated on either small molecules such as hormones, antibiotics and toxins or on the total synthesis of larger molecules like ribonuclease, leaving in comparative neglect the synthesis of active portions of molecules. Small sequences of proteins have been

shown for instance to exhibit Ca^{2+} binding (Leavis et al., 1978, Reid et al., 1980) and antigenic determinancy (Atassi, 1975). It is our contention that because of the small size and high activity of the cyanogen bromide fragment of Tn-I, this peptide is an excellent choice to help us relate the sequence of Tn-I to its biological function. We intend to demonstrate the feasibility of synthesizing and purifying an analog of this region and show that this analog behaves similarly to Tn-I but differently than other basic proteins which show inhibition. We will then construct a series of peptides that will allow us to define the shortest sequence that retains activity comparable to the cyanogen bromide fragment and thus define the actual region of Tn-I involved in this inhibition. These peptides will also allow us to examine the contribution to the inhibition by the side chains of some of the amino acids of the sequence and the possible complicating effect of charge on the α-amino and α-carboxyl groups of the peptide. Finally we will examine the effect of substitution of some of the amino acids in this region, as found in Tn-I from sources other than rabbit skeletal muscle and attempt to relate differences in activity to differences in sequence.

There is no doubt that studies of this kind are convenient, testing a part rather than the whole molecule. The question can be asked: is it meaningful? Does the study of a portion of a molecule really tell us anything about how that piece acts in a molecule? Of course there is no definite answer to this question but an intriguing possibility exists in the concept of exons. These are regions of DNA which are expressed and are flanked by regions that are not expressed called introns (Gilbert, 1978; Tonegawa et al., 1978). Exons may code for amino acid sequences that have specific functions and several exons may be involved in coding

for a single protein. These exons could then be shuffled to construct new proteins out of the functional domains of old ones. These exons are not necessarily large; the exon for the hinge region of the immunoglobulin heavy chain codes for 14 residues (Sakano et al., 1979) and that of the signal peptide of the immunoglobulin light chain contains the information for 19 residues (Tonegawa et al., 1978). If this hypothesis is correct the study of some peptides containing functional domains of their parent protein may turn out to be not only useful but natural.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. SOURCES OF CHEMICALS AND SOLVENTS

All chemicals and solvents were reagent grade. Diisopropylethylamine, pyridine and methylene chloride were redistilled prior to use. Boc-amino acids were purchased from Spinco Division Beckman Instrument Inc., Palo Alto, California, Protein Research Foundation, Japan and Bachem Fine Chemicals Inc., Marina Del Rey, California. Copoly(styrene-1%-divinylbenzene)chloromethyl resin (0.90 mmol Cl/g resin) and copoly(styrene-1%-divinylbenzene)benzhydrylamine-HCl resin (0.49 mmol NH_2/g resin) were purchased from Pierce Chemical Co., Rockford, Illinois and Protein Research Foundation, Japan respectively. Picric acid (Eastman Organic Chemicals, Rochester, New York) was dissolved in methylene chloride and dried over $MgSO_4$ before use (Hodges and Merrifield, 1975). Bio-Rex 70 (200 - 400 mesh sodium form) was purchased from Bio-Rad Laboratories, Richmond, California and the Sephadex G-10 (200 - 400 mesh) from Pharmacia (Canada) Ltd., Dorval, Quebec. Salmine was obtained from BDH Chemicals Ltd, Dorset, United Kingdom. Rabbit skeletal muscle was purchased from Pel Freeze Biologicals Inc., Rogers, Arkansas. ATP was purchased from Terochem Chemicals Ltd., Edmonton, Alberta. Rabbit skeletal α-tropomyosin was a generous gift from the lab of Dr. L.B. Smillie as was some of the rabbit skeletal Tn-I. The lab of Dr. C.M. Kay also generously provided some of the Tn-I.

B. AMINO ACID ANALYSIS, PEPTIDE AND PROTEIN QUANTITATION

The quantities of peptides, salmine and Tn-I used were determined from amino acid analyses after hydrolysis in 1 ml 6 N HCl in evacuated sealed tubes for 24 h at 110° C. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolysate was used to calculate the concentration of protein or peptide. Quantities of rabbit α-Tm, myosin, actin, skeletal S-1, cardiac S-1, skeletal Tn-I and cardiac Tn-I were determined on a Cary 118C spectrophotometer using $E^{1\%}_{280}$ and molecular weights as found in Table I.

C. EQUIPMENT

The peptides were synthesized on a Beckman peptide synthesizer Model 990. They were detected in the effluent from the Bio Rex 70 column with a Technicon auto analyzer with stream division for ninhydrin detection and in the effluent from the Sephadex G-10 column with a Schoeffel Spectroflow monitor and monochromator (SF 770 and GM 770), respectively. The peptide was cleaved from the resin support in a Type I HF apparatus with absorption cylinder from the Protein Research Foundation.

D. PROTEIN PURIFICATION

1. General

a. Cautions

Proteins can be very tricky molecules with which to work and purify. Muscle proteins are no exception. While there are several general precautions one should take, there is one that supercedes all others. A procedure should always be examined for what is not there. This process I have christened vacuum gleaning. Omissions may include such

TABLE I

Rabbit muscle protein extinction coefficients and molecular weights

Protein	$E^{1\%}_{280}$ cm^{-1}	Reference	Molecular Weight	Reference
Actin	11.0	Houk & Ue (1974)	42,000	Elzinga et al. (1973)
Myosin	5.88	Verpoorte & Kay (1966)	470,000	Lowey & Cohen (1961)
Cardiac S-1	7.5[a]	Wagner & Weeds (1977)	115,000	Taylor & Weeds (1976)
Skeletal S-1 (A1)	7.9	Yagi et al. (1967)	115,000	Weeds & Taylor (1975)
α-Tropomyosin	3.3	Woods (1969)	66,000	Stone et al. (1974)
Cardiac Troponin-I	4.37	Grand et al. (1976)	24,000	Grand et al. (1976)
Skeletal Troponin-I	6.6	Mani et al. (1974)	21,000	Wilkinson & Grand (1975a)

[a]Assumed.

technical details as flow rates, column sizes, the centrifugal force of a spin rather than the rpm and even pH. One common omission is whether the pH was readjusted or even checked after the addition of molecular species known to affect pH, e.g., ATP, EGTA and proteins. To emphasize the importance of these kind of details I merely note, that the addition of Tn-C to a 0.1 M $NaHCO_3$ buffer (pH=8.3) in the procedure for making the Tn-I affinity column, resulted in a pH drop of 2 units. Sample preparation is another hotbed of incomplete information. Before a protein was put on a column, how was it dissolved, what was its concentration and once again was the pH adjusted? Dialysis is often treated so cavalierly that the buffer dialyzed against is never mentioned. Finally some procedures may refer to papers that are clearly inappropriate. While this list is not exhaustive, hopefully, it will serve to sensitize others to the dangers in following procedures uncritically.

In general, all muscle protein preparations should be treated with great care. Due to their great sensitivity to metal ions all these studies were done with water which had been deionized in the building's mixed bed resin, distilled in glass in our lab and then deionized once again in a Barnsted demineralizer. Water which has undergone this procedure may sometimes be referred to as d^3 H_2O. Also because of metal ion sensitivity muscle preparations were allowed to come into contact with metal surfaces only when this was unavoidable, e.g. grinding and blending and then it was ensured that these surfaces were of stainless steel and that the time spent in contact with them was minimal. Unless otherwise indicated all extractions, preparations and centrifugations were carried out at 4°C, in a cold room. Rubber gloves were worn to eliminate contamination and subsequent degradation by

enzymes on the skin. Muscle preparations should not be subject to extremes of pressure. Stirring and vacuum filtrations should be performed as gently as possible to reduce denaturation due to frothing. Myosin is the most sensitive of all muscle proteins and as such these precautions are even more critical for it.

b. SDS gel electrophoresis

All SDS gel electrophoresis was done with 8% acrylamide 6 M urea gels using the procedure of Weber and Osborne (1969). Samples were dissolved in a solution containing 6 M urea, .03 M Na_2HPO_4, .02 M NaH_2PO_4 pH = 7.0, 1% SDS and 1% β-mercaptoethanol and heated at 60°C for 1 hr. Bromophenol blue dye (.05% in .04 M NaH_2PO_4, .06 M Na_2HPO_4 pH = 6.5) was used for tracking after it was made up as a 1% solution with the 6 M urea buffer. After electrophoresis, gels were washed in methanol/acetic acid/H_2O (1:1:8, v/v/v) for 1 h, stained with Coomasie Brilliant Blue P-250 (.25% solution in methanol/acetic acid/H_2O; 5:1:4, v/v/v) for 30 minutes and then destained in methanol/acetic acid/H_2O (1:1:8, v/v/v). Gels were scanned at 596 nm and the amount of protein was estimated, if required, from the peak area with reference to standard curves constructed for each protein.

2. Actin

Actin purification was carried out in two stages. The first involved the preparation from muscle of acetone powder which can be stored in the cold indefinitely. The second results in the purification of actin from acetone powder.

a. Acetone Powder

The procedure for making acetone powder has been attributed to Feuer et al., (1948). 1000 gm of frozen rabbit muscle was placed in a

watertight plastic bag and thawed by several changes of lukewarm water. All extractions are expressed in terms of this amount of muscle (e.g. 12 volumes, 1000 gm x 12 volumes = 12 litres). A scalpel was used to remove fat, membranes and blood vessels with the muscle kept in a vessel on ice when not being cleaned. The cleaned muscle was put through a meat grinder with 4.5 mm diameter holes and ground with as little pulverizing as possible. The ground muscle was suspended in 12 volumes of 4°C d^3 H_2O and stirred for 30 minutes. The muscle was allowed to settle for 30 minutes and the supernatant was taken off with suction or by decanting. The remaining mixture was further compacted by a spin of 4760 x g for 5 minutes in a DPR-6000 I.E.C. centrifuge. The residue was then alternately resuspended, stirred and centrifuged by a spin of 4760 x g for 10 minutes. The first suspension was done in 3 volumes of 0.4% $NaHCO_3$ which had been treated with Chelex 100 to remove metal ions and the pH adjusted to 8.0 with 5 M NaOH and stirred for 15 minutes. The second suspension was in 3 volumes of 0.1 M carbonate buffer, which was composed of .05 M $NaHCO_3$ and .05 M Na_2CO_3 (which had been treated with Chelex 100 and the pH adjusted to 8.0) and was stirred for 10 minutes. The third suspension was in 1.5 volumes of 0.2 mM $CaCl_2$ and stirred by hand for 2 minutes. The final residue was suspended in 1.5 volumes of 95% ethanol, 0°C, and stirred by hand for 2 minutes. The stirring time in this and all the following solvent extraction steps was critical to maximize water removal while minimizing protein denaturation. The mixture was poured through 4 layers of cheesecloth and then squeezed very hard to remove the bulk of the ethanol. This procedure was repeated with 1.5 volumes of acetone at -20°C three more times, then the residue was spread out on a cheesecloth in a fumehood

to dry. It was then stored at -20°C until ready to use. This procedure typically yielded 90 gm of very white fibrous material from 1000 gm of rabbit skeletal muscle. Yields were similar for rabbit muscle except that the powder had a greenish-brown color.

b. Actin

The second stage of actin purification was in essence the method of Spudich and Watt (1971). Buffer A consisted of 2 mM Tris base, 0.2 mM ATP, 0.5 mM DTT and 0.2 mM $CaCl_2$, which was adjusted to a pH of 8.0 with 5 M HCl. 10 gm of acetone powder was added to 20 volumes of Buffer A and stirred for 30 minutes at 4°C. This was centrifuged at 77,000 x g for 20 minutes. The supernatant was filtered under vacuum through Whatman 54 filter paper and then filtered sequentially through the following Millipore filters; SC (8 μm), SM (5 μm), SS (3 μm), and HAWP (.45 μm) under gentle vacuum. At this point the solution was clear. The solution was then made up to 2 mM $MgCl_2$ and 50 mM KCl with 1 M stock solutions to induce polymerization (G- to F-actin). This process was allowed to proceed for 2 hours at room temperature. At the end of this time enough solid KCl was added to make the solution 0.6 M KCl and this was stirred gently at room temperature for 2 hours. This mixture was centrifuged at 110,000 x g for 3 hours and the resulting pellets were homogenized in a hand homogenizer with approximately 20 ml of Buffer A. This was dialyzed against 1000 ml of Buffer A. This buffer was changed once every 12 hours, a total of 3 times. The G-actin which resulted from this dialysis was spun at 110,000 x g for 1 hour to clarify the solution and the absorbance was read at 280 nm with the last dialysate used as blank. This procedure typically yielded 100 mg of actin from 10 gm of either rabbit skeletal or rabbit cardiac acetone powder.

Any actin not used immediately was stored in the following manner. DTT was added to the solution to make it 1 mM in fresh DTT. The solution (5-10 mg G-actin/ml) was added slowly, 1 drop at a time into a beaker filled with liquid nitrogen. The resulting pellets were stored at -20°C. When the actin was to be used, it was thawed by dialyzing against fresh Buffer A. Actin stored by this procedure was used after periods up to four months without loss of activity in activating the myosin ATPase.

3. Myosin

a. Myosin

The myosin purification procedure followed very closely that of Wolodko and Kay (1975) which in itself was based on Mueller et al., (1964). The following procedure was used for both rabbit skeletal and cardiac myosin except for two deviations. To make skeletal myosin 100 gm of muscle were initially used and this was blended before extraction whereas cardiac myosin was made from 300 gm of muscle which had been minced in a meat grinder before extraction. The muscle was thawed in a watertight plastic bag by several changes of lukewarm water. A stainless steel scalpel was used to remove fat, blood vessels and membranes with the muscle kept on ice when not being cleaned. The muscle was cut into chunks approximately 3 cm x 1 cm and quickly rinsed with cold d^3 H_2O twice to remove blood. This step was more important in the cardiac preparation. The extracting buffer was the Guba Straub solution at 4°C which consisted of 0.3 M KCl, 0.1 M KH_2PO_4, .05 M K_2HPO_4 and the pH was brought to 6.5 with 5 M KOH. The skeletal muscle was blended in a Waring blender at top speed for 15 seconds with 120 ml of Guba Straub solution. This puree was then poured into 780 ml of Guba Straub solu-

tion which contained enough ATP to give the mixture a final concentration of 1 mM. The pH was not corrected. In contrast cardiac muscle chunks were ground in a precooled meat grinder with 4.5 mm diameter holes. The mince was added to 900 ml of Guba Straub solution which contained 1 mM ATP. Once again the pH was not corrected.

Occasionally the cardiac muscle was blended as above with no ill effects, all extraction solutions contained 0.5 mM PMSF. The muscle and extraction mixture was stirred for 15 minutes. While the low pH and short extraction time minimizes actomyosin extraction, a volume of d^3H_2O equal to 900 ml minus the volume of muscle was added to this mixture after 15 minutes to preferentially precipitate the actomyosin by reducing the ionic strength. This required 800 ml d^3H_2O for a skeletal muscle preparation which started with 100 gm of muscle. The mixture was strained through 2 layers of cheesecloth and centrifuged for 15 minutes at 4760 x g. Cardiac myosin was preferentially precipitated with 900 ml d^3H_2O. The mixture was centrifuged as above, the supernatant discarded and the precipitate was re-extracted as above, then preferentially precipitated with 400 ml d^3H_2O and centrifuged as above once more. From this point on the procedures were the same. The supernatant was again strained through cheesecloth and the volume measured. This was added to six times its volume of 2 mM β-mercaptoethanol solution. A white flocculent precipitate resulted. This was allowed to settle for 2-3 hours. If the yield was very high the precipitate settled only 1 to 2 cm. The clear supernatant was siphoned or decanted off and discarded. The remaining mixture was centrifuged at 4760 x g for 25 minutes. The resulting pellet was made up to 480 ml with concentrated Guba Straub solution, ATP and cold d^3H_2O to make a

solution 0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 1 mM ATP, pH 6.5. This was stirred gently for 15 minutes to dissolve the pellet and then spun for 15 hours at 48,000 x g. The supernatant was filtered through Whatman 541 filter paper, its volume measured and added to 10 volumes of d^3H_2O. The precipitate was allowed to settle for 20 minutes and spun at 4760 x g for 25 minutes. Pellets were dissolved with a concentrated solution of 1 M KCl and 1 M Tris-HCl, pH 7.0 and cold d^3H_2O was added to make a final volume of 480 ml. The resulting solution was 0.2 M KCl and 10 mM Tris-HCl, pH 7.0. The ionic strength was crucial because if it was any higher the purification resulting from the next step was low and if the ionic strength was any lower the yield decreased. The pH was also rechecked at this point and corrected if necessary. To this solution was added approximately 10 gm of DEAE cellulose and stirred for 10 min. The DEAE cellulose had been washed exhaustively with 0.5 M HCl, d^3H_2O to neutrality, 0.5 M KOH, d^3H_2O to neutrality and a solution of 0.02 M KH_2PO_4, 0.03 M K_2HPO_4, 0.2 M KCl, pH 7.0 and then stored at 4°C as a paste. The DEAE cellulose removed contaminating ATP and nucleoprotein. The mixture was filtered through 4 layers of cheesecloth and then treated with another 10 gm of DEAE cellulose and filtered. The cloudy solution was centrifuged at 4760 x g for 40 minutes to pellet the fine cellulose particles. The supernatant was filtered through Whatman 541 filter paper, the volume measured, pH adjusted to 6.5 and then added to 6 volumes of d^3H_2O. The solution was left for 15 minutes and then centrifuged at 4760 x g for 40 min. Occasionally the final precipitation was replaced by adding solid KCl to make the solution 0.5 M KCl and then adding an equal volume of saturated ammonium sulfate (pH = 7.0, 4°C). The solution was spun at 4760 x g for 20 min. Then

after either procedure used, the pellet was dissolved in 20 ml of 1 M KCl and made up to 40 ml with d^3H_2O. The solution was stirred gently for 20 min., dialyzed against one litre of 0.5 M KCl, 1 mM DTT for 2 changes of 8 hours each. The solution was centrifuged at 105,000 x g for 3 hours to clarify it. The A_{280}/A_{260} ratio, with final dialyzate as blank, was calculated. A ratio in the range of 1.7 to 1.8 was taken as indication that little ATP, nucleoprotein, or denatured myosin were present. Typical yields of myosin from 100 gm of rabbit skeletal and 300 gm of rabbit cardiac muscle were 140 mg and 200 mg respectively.

b. S-1

The procedure used for making myosin subfragment-1 was basically that of Weeds and Taylor (1975). The initial purification steps were identical to those involved in myosin purification until the second precipitation (the one after the 15 hour centrifugation) and the 25 minute centrifugation at a force of 4760 x g. The pellet resulting from this spin was taken up in approximately 100 ml of 0.5 M KCl, .05 M Tris-HCl, 1 mM EDTA, pH 7.0. The mixture was dialyzed against 1 litre of this buffer for 2 changes of 8 hours each. This was centrifuged at 48,000 x g for 2 hours and to the supernatant was added an equal volume of saturated $(NH_4)_2SO_4$, pH 7.0. The resulting mixture was stirred for 15 minutes. The $(NH_4)_2SO_4$ had been treated with 10 gm of Chelex 100 resin and filtered to remove heavy metal ions. The resulting precipitate was collected by centrifugation at 4760 x g for 15 minutes. The pellet was taken up in 50 ml of 2 M KCl and then made up to 100 ml with d^3H_2O and the pH was adjusted to 7.0 with 5 M HCl. This was dialyzed against 1 litre of 0.12 M NaCl, .013 M Na_2HPO_4, .007 M NaH_2PO_4, 1 mM EDTA, pH 7.0 solution for 3 changes of 8 hours each. The myosin precipitated as filaments. This was brought to room temperature with gentle stirring. Chymotrypsin, dissolved in less than 10 ml of the last dialysis buffer, was added to the myosin solution to a concentration of 0.05 mg/ml. The digestion was allowed to continue for 15 minutes and was terminated with 0.1 M PMSF in 95% ethanol such that it had a final concentration of 0.5 mM. The procedure was altered for cardiac myosin in that the concentration of both the chymotrypsin and the PMSF was doubled. The mixture was stirred for 15 minutes to allow complete inhibition to occur. Insoluble material was removed by centrifugation at 45,000 x g for 30

minutes. The supernatant was dialyzed for 2 changes of 8 hours each against 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3, pH 7.0. If precipitate appeared the mixture was centrifuged at 45,000 x g for 30 minutes. The supernatant was applied to a DEAE-52 cellulose column (2.8 x 45 cm) which had been equilibrated with the same buffer as was used in dialysis. The S-1 was eluted with a linear gradient made of 500 ml of the above buffer and 500 ml of the same buffer which had been made 0.2 M in KCl (Fig. 8a). Cardiac S-1 was eluted with the same gradient except the final buffer was 0.5 M KCl (Fig. 8b). The column was run at 38 ml/hr or 6.2 $ml/cm^2 \cdot hr$ and fractions were collected every 10 minutes. Peaks found by reading fractions at 280 nm were collected and concentrated on an Amicon ultrafiltration device equipped with a PM-10 filter. Cardiac S-1 was further concentrated with a 40-55% $(NH_4)_2SO_4$ precipitation and dialyzed with 2 changes of 8 hours each of 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3 buffer, pH 7.0. In this concentrated form the S-1 could be stored under a crystal of thymol with no loss of activity for three weeks if it was dialyzed against the ATPase assay buffer containing 1 mM DTT before use. In the interest of economy we found that the supernatant from the first extraction in the troponin preparation could be used as the starting material for the myosin preparation and S-1 with excellent yields. Typical yields from 100 gm of rabbit skeletal muscle and 300 gm of rabbit cardiac muscle were 60 mg and 40 mg of S-1 respectively. Typical acto-S-1 (A1) ATPase activities were 3.8 µmol PO_4/mg S-1 min. at a concentration of actin of .146 mg/ml. This preparation demonstrated a V_{max} of 28.4 S^{-1} compared to the value of 28 S^{-1} for Weeds and Taylor (1975).

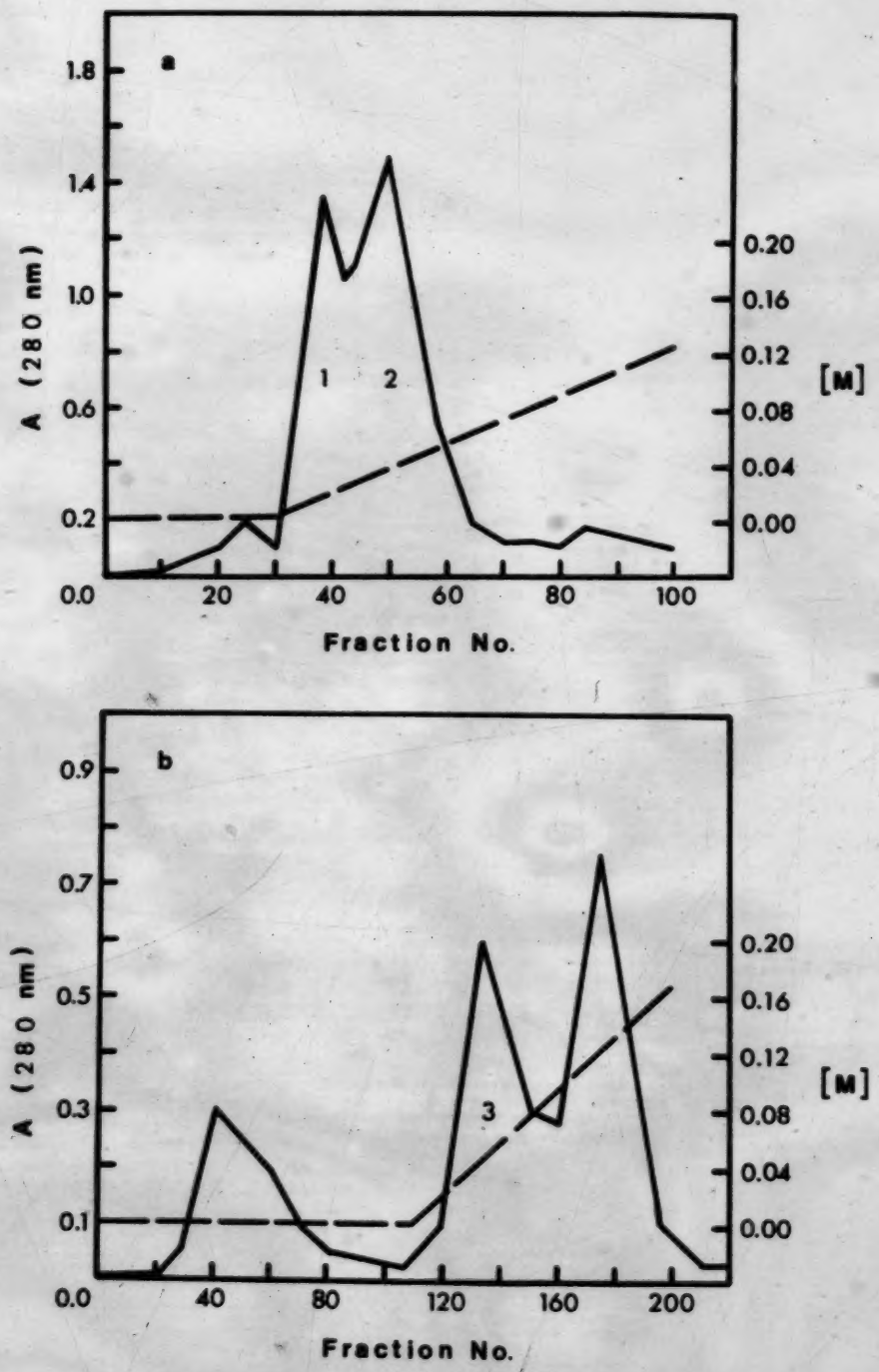


Fig. 8. DEAE-52 cellulose purification of rabbit skeletal and cardiac S-1.

(a) Rabbit skeletal S-1 profile, 1) S-1 (A1), 2) S-1 (A2).

(b) Rabbit cardiac S-1 profile, 3) S-1. [M] molarity of KCl as determined by conductivity. Conditions as in Procedure.

4. Troponin

This procedure was first used by Ebashi et al., (1968) but contains modifications of Staprans et al., (1972). 1000 gm of frozen rabbit muscle was placed in a watertight plastic bag and thawed by several changes of lukewarm water. A stainless steel scalpel was used to remove fat, blood vessels and membranes, the muscle was kept on ice when not being cleaned and then was cut into small chunks. The muscle chunks were homogenized in a Waring blender at top speed for 15 seconds with 1 volume (1000 ml) of Guba Straub solution (0.1 M KH_2PO_4, .05 M K_2HPO_4, 0.3 M KCl buffer, pH 6.5). The puree was mixed into 2 volumes of Guba Straub solution which had been made up to 1.5 mM ATP, 0.15 mM PMSF, the pH was uncorrected. The extraction was carried out with stirring for 10 minutes. The extraction was terminated by centrifuging at 4760 x g for 20 minutes. The supernatant can be used in myosin preparation, otherwise it was discarded. The residue was washed by stirring it with 3 volumes of .02 M KCl, 2 mM $KHCO_3$, for 10 minutes and then centrifuged at 4760 x g for 10 minutes. This step was repeated once. The volume of the pellet was measured and it was added to an equal volume of 0.8 M LiCl, 0.1 mM PMSF, 100 mM CH_3COONa buffer, pH 4.5 and the pH readjusted quickly to 4.5 with 5 M HCl. The suspension was extracted with constant stirring at 4°C for 2.5 hours and centrifuged at 4760 x g for 10 minutes. The supernatant was decanted and made up to a concentration of 50 mM with $KHCO_3$ and the pH taken up to 7.6 with 5 M KOH. After stirring for 10 minutes the pH was dropped to 4.5 with 5 M HCl to isoelectrically precipitate tropomyosin. This was gently stirred for 10 minutes and centrifuged at 4760 x g for 10 minutes. The supernatant was decanted and adjusted to a pH of 7.6 with 5 M KOH. If pure troponin was

desired the ammonium sulfate fractionations were carried out differently than if the troponin was to be used to prepare Tn-C. In the latter case to maximize yield, the total volume of supernatant was determined and ammonium sulfate added to bring the solution to 37.5% saturation at 0°C, stirred for 10 minutes and then centrifuged at 4760 x g for 25 minutes. The second cut was taken at 60% and spun in the same fashion. When trying to ensure maximum purity cuts were made at 45%, 50%, 55% and 60%. Although the 50 - 55% fraction usually contained the bulk of pure troponin, 8% polyacrylamide, 1% SDS, 6 M urea gels were usually run on each fraction. The fractions were dialyzed exhaustively against a solution of 15 mM β-mercaptoethanol, 2 mM HCl, regardless of where the cuts were made and the solution was freeze dried and stored at 4°C. Typical yield for a 37.5% to 60% ammonium sulfate fraction was 1.2 gm of freeze dried powder from 500 gm of frozen rabbit skeletal muscle.

5. Troponin-C

This procedure was based on that of Eisenberg and Kielley (1974). The crude freeze dried troponin was dissolved in 100 ml of 8 M urea, 1 mM DTT, 10 mM EGTA, 50 mM Tris-HCl buffer, pH 8.0 so that the final concentration was 10 mg/ml. The pH was readjusted with 5 M KOH to 8.0 after addition of troponin and stirred gently for 10 minutes. This was dialyzed for several hours against 1 litre of the above buffer at room temperature. The solution was loaded at a flow rate of 20 ml/hr on to a DEAE Sephadex A-25 column (2.8 x 45 cm), which had been equilibrated with same buffer. The column was eluted with 250 ml of starting buffer (8.0 M urea, 15 mM β-mercaptoethanol, 10 mM EGTA, 50 mM Tris-HCl, pH 8.0) followed by 100 ml of the same buffer containing 0.1 M KCl to

elute a peak which is primarily Tn-T. Fractions of 15 min. were collected. The column was then developed with a linear salt gradient made from 400 ml of starting buffer containing 0.1 M KCl and 400 ml of starting buffer containing 0.6 M KCl. The fractions were read at 280 nm and the peak of Tn-C was eluted at approximately 0.38 M KCl (Fig. 9). The pooled Tn-C peak was dialyzed exhaustively against 15 mM β-mercaptoethanol solution and freeze dried. A typical yield of Tn-C was approximately 185 mg of freeze dried material (100 mg by absorbance measurement. This was isolated from 1 gm of crude troponin.

6. Troponin-I

This Tn-I affinity column purification followed very closely that of Syska et al., (1976). 40 gm wet weight of Sepharose 4B was washed extensively with d^3H_2O and floated to remove fines. It was then made up to a volume of 50 ml with d^3H_2O and was placed in a 250 ml round bottom flask with a stirring bar. The pH was adjusted to 11.0 with 5 M NaOH and 4 gm of CNBr were added. The pH was maintained in the range of 11.0 to 11.5 by the addition of 5 M NaOH from a pasteur pipet by hand and the temperature kep below 25°C by addition of d^3H_2O ice chips during the course of the reaction. When the pH ceased to change significantly the activated Sepharose was washed with 1 litre of cold d^3H_2O and then 1 litre of cold 0.1 M $NaHCO_3$, pH 8.3. The resin was suspended in 10 ml of 0.1 M $NaHCO_3$, pH 8.3 and added to a plastic vial. Tn-C (100 mg by absorbance) was added to 5 ml of 0.1 M $NaHCO_3$, pH 8.3 containing 5 mM $CaCl_2$ (the $CaCO_3$ was a precipitate until the Tn-C was added). The pH was measured and adjusted with 5 M KOH to 8.3. The reaction was initiated by adding the Tn-C solution, 20 mg/ml, to the resin in the plastic vial and shaking it in a 105° rocking shaker at

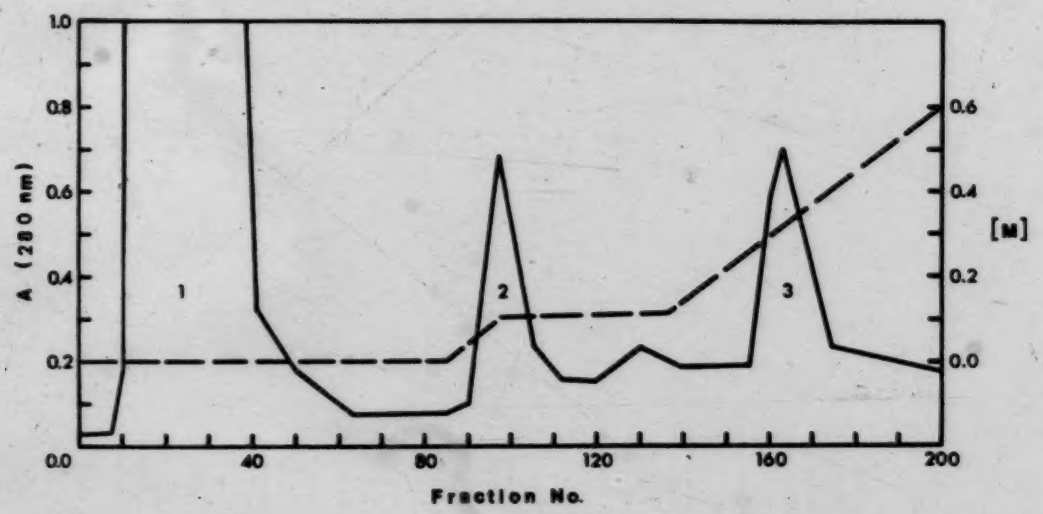


Fig. 9. DEAE Sephadex purification of Tn-C.
(1) Peak by gel electrophoresis shown to contain both Tn-I and Tn-T.
(2) Peak consisting primarily of Tn-T.
(3) Peak consisting exclusively of Tn-C. [M] molarity of KCl as determined by conductivity. Conditions as in Procedure.

4°C for 20 hours followed by 2 hours at 20°C. The Tn-C Sepharose conjugate was washed with 200 ml of cold d^3H_2O and 200 ml of 0.1 M $NaHCO_3$, pH 8.3. This washing was repeated 4 times. By comparing the absorbances of the washes to the initial coupling mixture we calculated that approximately 75 mg of Tn-C had coupled. The resin was equilibrated with 8 M urea, 15 mM β-mercaptoethanol, 1 mM $CaCl_2$, 50 mM Tris-HCl buffer, pH 8.0 (9 M urea when isolating cardiac Tn-I). A column (2 x 9 cm) was packed with the resin and 100 ml of the equilibration buffer was run through the column at 18 ml/hr and was followed by 50 ml of equilibration buffer containing 0.5 M KCl to ensure no non-covalently bound Tn-C remained. 50 ml of equilibration buffer was flushed through the column for reequilibration before sample application.

The sample from which Tn-I was to be isolated was prepared in one of two ways. In the first, used for skeletal Tn-I, the peak from the DEAE Sephadex A-25 purification which contained a mixture of Tn-I and T (eluted with starting buffer) was pooled and dialyzed exhaustively against 2 mM HCl, 15 mM β-mercaptoethanol and freeze dried. 50 mg of this powder was dissolved in 2.5 ml column buffer (8 M urea, 15 mM β-mercaptoethanol, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0) and the pH readjusted to 8.0 with 1 M KOH. The sample was dialyzed against the same buffer for several hours to ensure equilibration and dissocation.

The second method used for cardiac Tn-I, was that of Grand et al., (1976). We modified their procedure as follows. The homogenate extraction mixtures were stirred for 30 minutes, filtered through one layer of cheesecloth, combined with affinity resin and overhead stirred (no stirring bars) for 30 minutes. The mixture was centrifuged at 10,825 x g for 15 min. The supernatant was decanted, the resin resus-

pended in 200 mls of 9 M urea affinity buffer and centrifuged at 1500 x g for 15 min. The supernatant was decanted, resin packed into a column and the rest of the procedure was the same.

At this point regardless of method the sample was loaded onto the column, fractions were collected every 15 minutes and column buffer was flushed through until the absorbance of the column effluent was the same as that of the buffer. The Tn-I was eluted from the column with column buffer containing 10 mM EGTA (pH readjusted to 8.0 with 5 M KOH). The Tn-I peak was located by reading the fractions at 280 nm (Fig. 10), pooled, dialyzed against 15 mM β-mercaptoethanol, 1 mM HCl, pH 2.5 and then freeze dried. Typical yields of Tn-I were 25 mg of freeze dried powder from each column run (15 mg of Tn-I by absorbance measurements).

E. ACTOMYOSIN AND ACTO-S-1 ASSAYS

1. Assay of Inhibitory Activity

a. Tn-I

Tn-I is notorious for its solubility problems. We have circumvented these in the following manner: the Tn-I was dissolved in 1 mM HCl pH 2.5. Its concentration was 10 mg lyophilized powder/ml. Enough solid KCl and urea were added to make the solution 0.5 M and 8 M respectively. Aliquots of 1 M Tris-HCl buffer, pH 7.6 were added to make the solution 10 mM and the pH was adjusted to 7.6. The solution was dialyzed overnight against two changes of 500 ml each of a solution which was 2 mM DTT, 0.5 M KCl, 10 mM Tris-HCl, pH 7.6. The resulting dialysate had a faint precipitate which was removed by a spin of 1 min at 9380 x g on a Beckman 152 Microfuge or by filtration through Millipore HAWP (0.45 μm) filters. Tn-I was assayed in the following manner

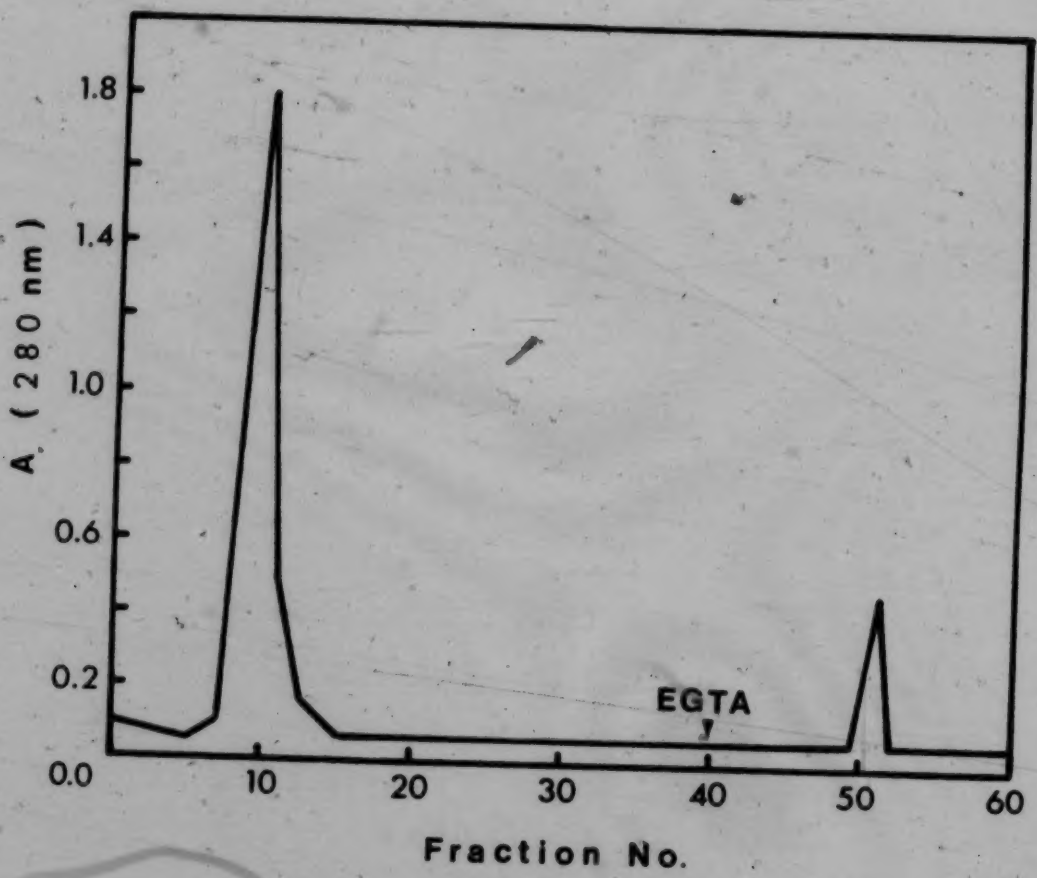


Fig. 10. Purification of Tn-I. Tn-C affinity column purification of cardiac or skeletal Tn-I. EGTA indicates point at which buffer containing EGTA was started. Conditions as in Procedure.

to further minimize solubility problems. The skeletal Tn-I was aliquoted into a mixture containing 180 µg acto-S-1 (A1) (1 part actin to 1 part S-1 (A1) by weight, protein concentration 0.3%, 10 mM Tris-HCl pH 7.6), 135 µg Tm and enough of the above Tn-I dialyzing buffer such that after addition of 3 ml of reaction mixture (see below) the KCl concentration resulting from the Tn-I solution and the dialysis buffer would be 7 mM. When the effect of increasing the actin concentration was examined the mixture contained 3.5 times as much actin and tropomyosin while S-1 was kept constant, i.e. 315 µg actin, 473 g Tm, 90 µg S-1. The assay was initiated by adding 3 ml reaction mixture which consisted of 2.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM EGTA, 10 mM Tris-HCl pH 7.6.

b. Peptides

The peptides had no solubility problems and were assayed by adding them to 3 ml of reaction mixture identical to the above except it was 7 mM KCl and contained 135 µg Tm (473 µg Tm for assays at high [actin]). The reaction was initiated by adding 450 µg actomyosin (1 part actin to 4 parts myosin by weight, total protein concentration 0.3%, 0.3 M KCl, 10 mM Tris-HCl pH 7.6) or 180 µg skeletal acto-S-1 (A1) (1 part actin to 1 part S-1 (A1) by weight, total protein concentration 0.3%, 10 mM Tris-HCl pH 7.6) or 270 µg cardiac acto-S-1 (1 part actin to 1 part S-1 by weight, total protein concentration 0.3%, 10 mM Tris-HCl pH 7.6).

2. Phosphate Determination

Due to the large number of phosphate determinations to be performed we chose as our method that of Fiske and Subbarow (1925). Although everyone cites this paper it seems likely that the details of

their procedures differ a great deal from the original method. We allowed the ATPase to continue for 10 minutes, 15 minutes for cardiac myosin or S-1 assays, and then quenched the reaction by adding 3 mls of 15% TCA to precipitate the protein. This mixture was filtered through Whatman 54 filter paper and 2 ml aliquots were taken from each sample and pipetted into 1 ml of 2.5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 5 N H_2SO_4. To each of these was added on a 30 second stagger 1 ml of ANSA solution (5.7 gm $NaHSO_3$, 0.1 gm 4-amino-3-hydroxy-1-napthelenesulfonic acid, 0.2 gm Na_2SO_3 made up to 200 ml with d^3 H_2O).

This solution was unstable and was made fresh before each set of assays. The resulting blue color was quantitated by reading the absorbance at 660 nm after 10 minutes. All reactions were carried out in 18 x 150 mm culture tubes and to insure no contaminating phosphate was present, new tubes were used for each assay.

F. PEPTIDE SYNTHESIS AND PURIFICATION

1. Attachment to the Solid Support

Two methods of attaching the carboxyl terminal amino acid to the solid support were used. In the first method the carboxyl terminal amino acid was esterified as the Boc-amino acid cesium salt to the co-poly-(styrene-1%-divinylbenze) chloromethyl resin (0.90 mmol Cl/gm of resin) according to the method of Gisin (1973). It should be noted that reaction of 2.5 times excess Boc-amino acid cesium salt for 24 h at 50°C gave substitutions that ranged between 0.6 to 0.85 mmol/gm resin for every amino acid except Boc-Arg (Tos). This amino acid gave a substitution of only 0.18 mmol/gm of resin under the same reaction conditions even though the reaction time was extended to 120 h. This

low yield was presumably due to steric effects. The second method of attachment was used when a carboxyl terminal amide was desired on the peptide. In that case 0.32 mmol of Boc-amino acid and DCC/gm of resin were added to the copoly (styrene-2%-divinylbenzene) benzhydrylamine resin (0.45 mmol NH_2/gm of resin). This coupling resulted in a substitution of approximately 0.28 mmol/gm of amino acid resin. Excess amino groups were terminated by a 90 min treatment with acetic anhydride/benzene/pyridine (1:3:3, v/v/v, 25 ml/gm of amino acid resin) after prewashing the resin for 5 min with the same solution.

2. Substitution Level Strategy

It has been shown that optimum substitution levels of resin for peptide synthesis range from 0.1 to 0.3 mmol peptide/gm of resin. Therefore all amino acid resins which had been produced by cesium salt esterification were reduced to this level by adding 0.3 mmol of the second amino acid and DCC/gm of amino acid resin. This resulted in a substitution level of 0.25 mmol/gm of peptide resin. The remaining free amino groups were terminated with acetic anhydride as above except that the reaction time was 60 min.

3. Synthesis

All amino groups were protected at the α-amino position with the Boc group and the following side chain blocking groups were used: Arg (Tos), Lys (2-ClZ), Asp (O-benzyl) and Thr (benzyl). Boc-amino acids (2.25 mmol, 3 equivalents) in 16 ml of CH_2Cl_2 were added to the peptide-resin (3 gm) followed by 9 ml of DCC (2.25 mmol). Boc Arg (Tos) was dissolved in DMF/CH_2Cl_2 (1:5, v/v) because of low solubility in CH_2Cl_2 alone. Each coupling was done twice and stirred for 90 min. Boc groups were removed at each cycle with 55 ml of 50% TFA/CH_2Cl_2

(v/v) for 25 min. Neutralizations were carried out with 50 ml of 5% DEA/CH_2Cl_2 (v/v).

Synthesis was carried out automatically on a Beckman 990 peptide synthesizer using the following program for the attachment of each amino acid: 50% TFA/CH_2Cl_2, 1 min; 50% TFA/CH_2Cl_2, 25 min; CH_2Cl_2, 4 times, 1 min; Isopropanol, 2 times, 1 min; CH_2Cl_2, 8 times, 1 min; 5% DEA/CH_2Cl_2, 3 times, 2 min; CH_2Cl_2, 6 times, 1 min; Boc-amino acid, 5 min; DCC, 90 min; CH_2Cl_2, 3 times, 1 min; Isopropanol, 2 times, 1 min; CH_2Cl_2, 2 times, 1 min; Isopropanol, 2 times, 1 min; CH_2Cl_2, 6 times, 1 min; 5% DEA/CH_2Cl_2, 3 times, 2 min; CH_2Cl_2, 6 times, 1 min; Boc-amino acid, 5 min; DCC, 90 min; CH_2Cl_2, 3 times, 1 min; Isopropanol, 2 times, 1 min; CH_2Cl_2, 2 times, 1 min; Isopropanol, 2 times, 1 min; CH_2Cl_2, 6 times, 1 min. The CH_2Cl_2 and isopropanol washes were 75 mls and 50 mls respectively.

4. Cleavage of Peptides from Resin

The protected peptide resin was dried under high vacuum at 25°C for 20 hours. The cleavage of the resin and removal of blocking groups was carried out by placing 1 gm of peptide resin and 1 ml anisole (as a scavenger for cations) in the HF reaction vessel. The vessel is then cooled to -78°C with liquid nitrogen and 9 ml of HF were distilled under vacuum into the reaction vessel. The temperature was then maintained at 0°C with an ice/water bath for 60 min. The HF and bulk of the anisole were removed under vacuum at 0°C and the residual anisole and byproducts were extracted with ether. The peptide was dissolved in 20 ml TFA and filtered to remove resin. The TFA was evaporated and the peptide was dissolved in water and lyophilized.

5. Purification of Peptide

The crude peptide preparation was dissolved in 2 ml of pyridine-acetate buffer (1.0 M in pyridine, pH 5.0) and applied to a column (1.9 x 50 cm) containing Bio Rex-70 resin and eluted with a linear gradient from 1500 ml of 1 M pyridine-acetate, pH 5.0 to 1500 ml of 2 M pyridine-acetate, pH 5.0 at a flow rate of 60 ml/h at 50°C. The column effluent was stream split at a rate of 6.0 ml/h to a Technicon auto analyzer for ninhydrin detection of the peptide. Only one major peak was detected for all peptides and its fractions were pooled and lyophilized. This fraction was dissolved in .05 M NH_4HCO_3 pH 8.3 and eluted from a Sephadex G-10 column (1.5 x 110 cm) with the same buffer at 16 ml/h. The peptides were detected in the effluent by monitoring at 230 nm, pooled and lyophilized.

6. Resin Hydrolysis

Approximately 10 mg of resin was placed in a pyrex test tube and 2 ml conc. HCl, 1 ml phenol (88%) and 1 ml acetic acid were added to this. The tube was evacuated, sealed and heated for 24 h at 110°C. The tube was opened and the contents filtered through glass wool in a pasteur pipette to remove resin. The glass wool was washed 3 times with 5 ml 1 N HCl and 3 times with 5 ml H_2O. The liquid was evaporated to dryness at 50°C in a rotary evaporator and the residue was taken up in 15 ml H_2O and transferred to a 50 ml separatory funnel. This was extracted 3 times with 15 ml of $CHCl_3$ and the collected $CHCl_3$ washes were back extracted with 15 ml H_2O. The H_2O washes were combined and evaporated to dryness. The residue was dissolved in pH 2.2 buffer or water and the amino acid analysis performed on a Durrum D-500 amino acid analyzer.

G. F-ACTIN BINDING AND CENTRIFUGATION

The conditions used to examine the binding of F-actin and tropomyosin are the same as those used in the reaction mixture to test ATPase activity except that only 2 ml of reaction mixture were used, myosin was not included to avoid the consequent hydrolysis of ATP and formation of rigor complex, and one F-actin, Tm binding study was carried out in the presence of 5.0 mM $MgCl_2$ rather than 2.5 mM $MgCl_2$. The proteins were allowed to associate at room temperature for 15 min and then, if a visible precipitate had formed the reaction mixture was spun at 3700 rpm (1500 x g) for 15 min on an MSE benchtop centrifuge. The supernatant from these mixtures and the other reaction mixtures was spun at 25,000 rpm (37,000 x g) for 3 h on a Beckman model L using a 50 rotor. The pellets and previously collected precipitate were run on 1% SDS 6 M urea gels and the amount of each protein was calculated from the peak area of the gel by reference curves constructed for each protein.

CHAPTER III

INITIAL CHARACTERIZATION

A. CHEMICAL CHARACTERIZATION

1. Synthetic Strategy

The first synthesis (peptide I, Table II) was carried out to prove that the synthesis of a peptide closely resembling that of the natural sequence was feasible. The sequence differed from that of the sequence of the inhibitory cyanogen bromide fragment (Syska et al., 1976; Table II) by the deletion of residues 96 and 97 and the substitution of an alanine for homoserine at position 116. These changes made the synthesis simpler but did not result in the loss of any of the basic residues, which were thought to be important to the inhibitory activity of the fragment. Furthermore the amino acid at 116 in the parent protein is methionine so the cyanogen bromide fragment was itself an analog.

Since many analogs were to be constructed, we attempted to determine how well the synthesis progressed. Peptide I was constructed using 3 couplings of 1.75 equivalents of DCC and Boc-amino acid each. To determine the success of each coupling we took samples of peptide-resin at various points in the synthesis and subjected them to HCl-Phenol-Acetic acid hydrolysis. To find this coupling efficiency it was not necessary to sample after each coupling. Instead it was only necessary to sample just before an amino acid was to be coupled which was already present in the peptide and the amount of that amino acid had not been determined by a previous hydrolysis. For example in the sequence NH_2-Leu-Arg-Val-Arg-Ala-COOH the first sample would be after Ala was added to the resin the second after Val (before the second Arg after the last

TABLE II

Sequences of CNBr fragment of rabbit skeletal Tn-I; homologous rabbit cardiac region, homologous rabbit slow Tn-I region and synthetic peptides

	96	100	110	112	113	116
CNBr Fragment	NH_2 Asn Gln Lys Leu Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Pro Leu Arg	—	—	—	Arg Val Arg Hse OH	
Peptide I	NH_2 Lys	—	—	Arg	—	Ala OH
II		NH_2 Asp	—	Arg	—	Ala OH
III			NH_2 Gly	—	Arg	— Ala OH
IV			NH_2 Gly	—	—	Ala OH
V			NH_2 Gly	—	—	Arg NH_2
VI			NH_2 Gly	—	—	Val NH_2
VII			NH_2 Gly	—	Arg NH_2	
VIII			Ac Lys	—	—	Arg NH_2
IX			NH_2 Lys	—	—	Arg NH_2
X			Ac Phe	—	—	Arg NH_2
XI			NH_2 Phe	—	—	Arg NH_2
XII			NH_2 Lys	—	—	Arg OH
XIII			NH_2 Lys	—	Arg	— Arg NH_2
XIV			NH_2 Gly	—	Leu	— Ala OH
XV			NH_2 Gly	— Thr	— Arg	— Ala OH
XVI			NH_2 Gly	— Thr	— Leu	— Ala OH
Cardiac Sequence	Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu Arg	—	—	Leu Arg	Val Arg Ile	—
Slow Sequence	Lys Leu Lys Val Leu Asp Leu Arg Gly Lys Phe Lys Arg Pro Pro Leu Arg	—	—	—	Arg Val Arg Val	—

At the N terminal NH_2 indicates a free α-amino group and Ac indicates an acetyl group. At the C terminal OH indicates a free α-carboxyl group and NH_2 indicates an amide group.

sampling). These resin samples were subjected to HCl-Phenol-HAc hydrolysis. The results for each hydrolysis were normalized by making the value for Val equal 1.00. Val was chosen because it was reasonably stable to hydrolysis and it was the first amino acid of the peptide not present in excess, Ala was purposely present in excess and was not reported (Substitution level strategy, Ch II). The results of the six hydrolyses and the interpolated values calculated for each amino acid are shown in Table III. Examination of the results showed a decrease of amino acids added later to the growing chain. If the peptide had been synthesized with 100% efficiency these numbers would all be 1 $\pm$ 5% for the accuracy of the amino acid analyzer. That this is not the case indicates that premature termination may have occurred and this is responsible for the decreased yield.

While Table III indicates that 50% of the growing peptide chains contain residue 98 they also gave some clues as to the problem areas of the synthesis. In particular it appeared that the region Arg 113 to Val 115 and the coupling of Lys 107 to Arg 108 were responsible for 72% of the reduction in yield.

These results were interesting for delineating problem areas but they also suggested that 3 couplings of 1.75 equivalents apiece were not feasible for this synthesis. A 47% completion, while unacceptable, does represent a yield of not less than 97.5% on the average for each of 18 steps. The difference between this figure and 100% is the result of termination and incomplete coupling. Premature cleavage might also have occurred and although not detectable by this method it has been shown to account for losses of 2.3% for a 1 hr cleavage with 50% TFA (Erickson and Merrifield, 1976).

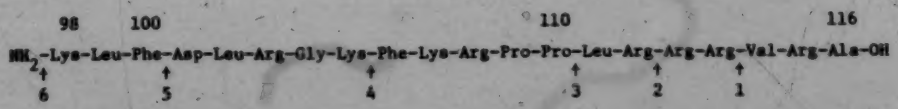
TABLE III

Progress of the synthesis of peptide I

Synthesis order	Sequence No.	Amino Acid	Hydrolysis 1	2	3	4	5	6
1	117	Ala						
2	116	Arg	0.89	0.89	0.89	0.89	0.89	0.89
3	115	Val	1.00	1.00	1.00	1.00	1.00	1.00
4	114	Arg		1.6	1.6	1.6	1.6	1.6
5	113	Arg						
6	112	Arg			0.81	0.81	0.81	0.81
7	111	Leu			0.80	0.85	0.85	0.85
8	110	Pro				N.D.	N.D.	N.D.
9	109	Pro						
10	108	Arg				0.80	0.80	0.80
11	107	Lys				0.64	0.64	0.64
12	106	Phe				0.61	0.63	0.63
13	105	Lys					0.63	0.63
14	104	Gly					0.63	0.63
15	103	Arg					0.73	0.73
16	102	Leu					0.62	0.62
17	101	Asp					0.56	0.54
18	100	Phe						0.51
19	99	Leu						0.49
20	98	Lys						0.47

Sampling points:

Peptide I



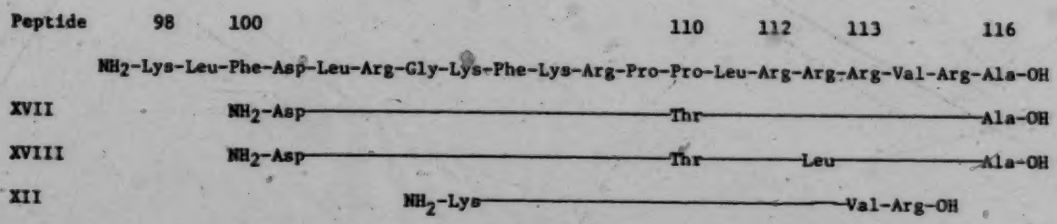
N.D., Not determined.

To rectify the low yields and reduce the impurity of the synthesis we examined the effect of altering the equivalents added and the number of couplings. The results of these manipulations are found in Table IV. This table contains the results of amino acid hydrolyses of the resin after the synthesis was finished. These were normalized by adjusting Val to 1.00 and each value was the amount of amino acid divided by the number of times that amino acid was found in the peptide. Inspection of the residues found only at the end of the synthesis e.g., Asp, Gly and Phe show that a dramatic increase in yield resulted from altering the synthesis from strategy 1 (3 couplings of 1.75 equivalents each) to strategy 2 (1 coupling of 1.5 equivalent and 2 couplings of 3.0 equivalents). Synthesis by strategy 2 went to 80% completion after 14 couplings (Gly) compared to 63% for strategy 1. Substantially the same results were achieved by 2 couplings of 3 equivalents and in the interest of economy of time and cost, this procedure was used for all subsequent syntheses. An intriguing demonstration of the importance of concentration is exhibited by comparing the yield of strategy 1 in Table IV and that of strategy 3 for Phe. The value of 0.57 contained in Table IV for Phe in strategy 1 is actually the average of Phe 106 and Phe 100. Table III reveals the actual value for Phe 106 is 0.63. The value for strategy 4 is 0.78 which is a good improvement when one considers that the total number of equivalents used in both strategies was similar, 5.25 for strategy 1, 6.0 for strategy 4.

That the yields increased merely by altering the equivalents suggested that this increase was not due to the elimination of premature cleavage which would be unaffected by this alteration. The most likely explanation for this reversible loss was that a competing terminating

Table IV

Effect of equivalence and coupling number on peptide yield



Amino Acid	Peptide I	Peptide XVII	Peptide XVIII	Peptide XII
	Strategy 1	Strategy 2	Strategy 2	Strategy 3
Lys	0.58	0.79	0.72	0.89
Arg	0.90	1.04	0.93	N.R.
Asp	0.56	0.83	0.72	N.P.
Pro	N.D.	N.D.	0.90	N.D.
Thr	N.P.	0.78	0.83	0.95
Gly	0.63	0.81	0.80	N.P.
Ala	N.R.	N.R.	N.R.	N.P.
Val	1.00	1.00	1.00	1.00
Leu	0.65	0.94	0.97	1.01
Phe	0.57	0.92	0.81	0.78

Strategy:
1. 1.75 equivalents 3 couplings
2. 1.5 equivalents 1 coupling, 3.0 equivalents 2 couplings
3. 3.0 equivalents 2 couplings

N.D. Not determined
N.P. Not present
N.R. Not reported

reaction was occurring which only became significant when the coupling reaction was slow. This would explain the large drops in yield where steric considerations probably slowed the coupling reaction. Furthermore increasing the equivalents would have driven the reaction towards completion lessening the extent of termination and resulting in the improved yield.

2. Peptide Purification

After demonstrating the feasibility of synthesizing a sequence resembling the actomyosin ATPase inhibitory region of Tn-I it was necessary to devise a purification procedure. While many ion exchange resins were available the choice of one suitable for this purification was complicated by the highly basic nature of this peptide sequence. Even relatively weak cation exchangers bound the peptides so strongly that very high ionic strength was necessary to elute them. The peptides were so small that any attempt to dialyze them would have resulted in substantial losses of peptide. The concentration of salt was so high and the peptide molecular weight so low that attempts to separate the salt from peptide on a gel-exclusion column failed. To overcome these problems we used a pyridine-acetate gradient to elute the peptide from Bio-Rex 70. Bio-Rex 70 is a weakly acidic carboxylic cation exchanger with an acrylic polymer lattice. To maximize resolution we wanted to run the column at 50°C and Bio-Rex 70 is an excellent choice for this as it has good thermal stability up to 100°C. The bulk of the pyridine-acetate was removed by lyophilization and the remainder removed by Sephadex G-10 chromatography. The resolution capable with this procedure is demonstrated by Figure 11. Here three similar peptides were chromatographed together and show excellent separation. The separation of these peptides was

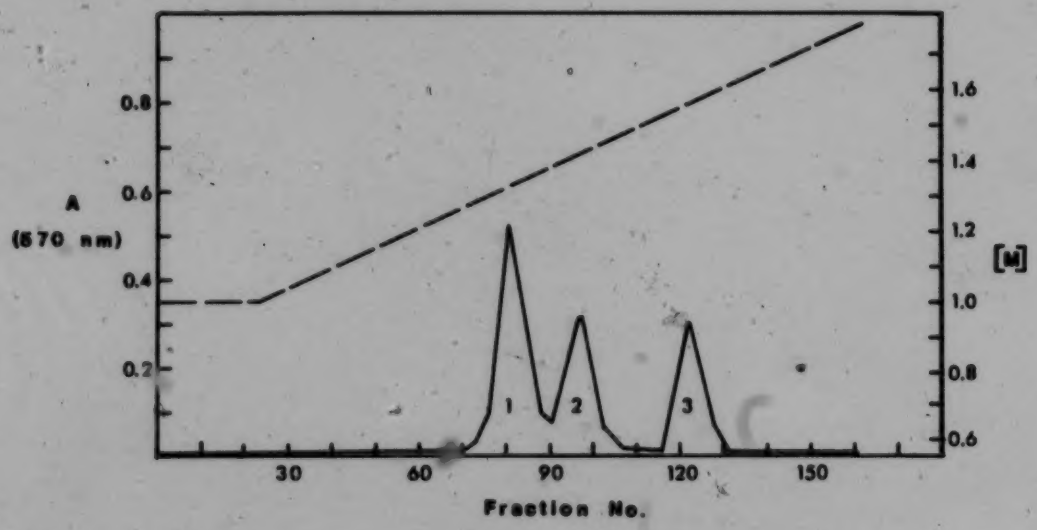


Fig. 11. Resolving power of Bio Rex 70 column. Chromatographic separation of three synthetic peptides differing in the number of basic amino acid residues from 5 to 7 on Bio Rex 70. The effluent was monitored by automatic ninhydrin analysis at 570 nm (——). The gradient profile is indicated as molarity of pyridine as determined by conductivity (- - -). Conditions as in Procedure.

Peak	Peptide	Sequence
1	VI	H_2N-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-NH_2
2	V	H_2N-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-NH_2
3	XIII	H_2N-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Arg-Val-Arg-NH_2

dependant on their differences in net charge and it was expected that the majority of termination or deletion peptides arising from the synthesis would vary by at least one basic residue due to the preponderance of these residues in the peptides (as high as 7 basic residues out of 12).

The actual elution profile of a typical synthetic peptide is revealed in Fig. 12. There were two peaks, one minor and one major. This major peak contained peptide XII (Table II). The minor peak was predominantly composed of arginine and valine, residues found at the carboxyl terminal and probably representing short failure sequences. The purity of peptides prepared in this manner is demonstrated in Table V, which compared the amino acid analysis of peptide I on the resin and after purification. This indicated the peptide was pure within the limits for the amino acid analyzer $\pm$ 5% after chromatographic purification. The amino acid analyses of all peptides used in this study are reported in Table VI.

3. Peptide Yields

We have followed peptide XII from its synthesis to its purification and determined the yield after each step (Table VII). The first residue coupled to the resin was Arg and the penultimate was Phe so we have expressed the % completion as the ratio of Phe to Arg derived from an hydrolysis of the resin, 79.6%. The peptide resin was cleaved with HF and washed with TFA. The TFA washes were analyzed to give the % cleavage of the completed peptide, 74.5%. The purification, as mentioned previously, resulted in two peaks. The amount of pure peptide was calculated as the amount of Val in the major peak divided by the total in both peaks. This number, 70% is in good agreement with that of 79.6%, which is the closely related percentage of completion. Taking into

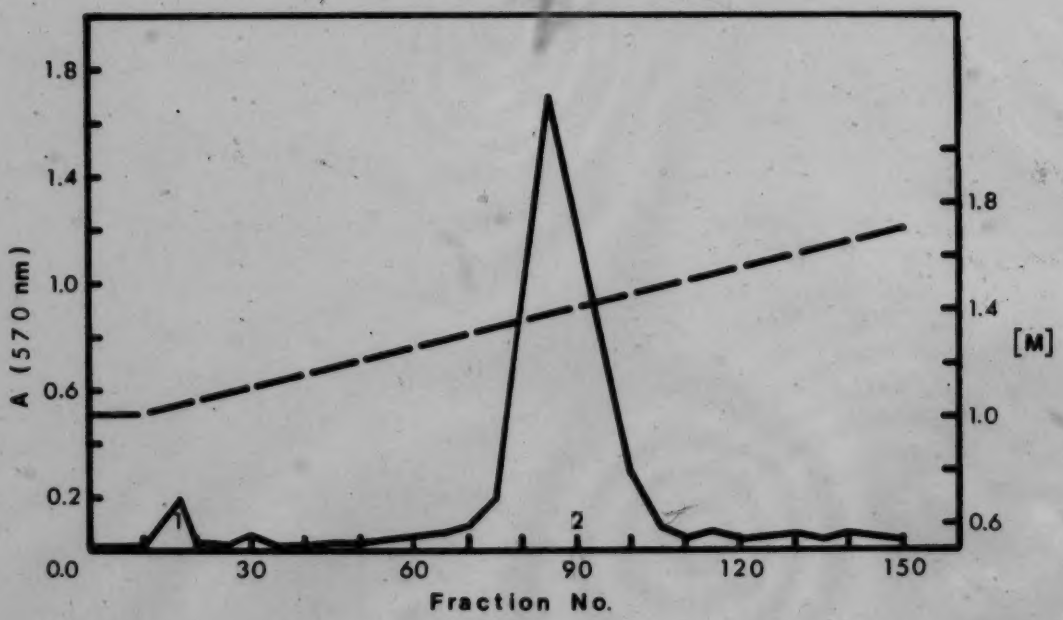


Fig. 12. Elution profile of purification of peptide XII on Bio Rex 70. Effluent monitored and gradient indicated as in Fig. 11.
1) Impurities, primarily composed of termination peptides
2) Pure peptide XII. Conditions as in Procedure.

TABLE V

Comparison of peptide I on resin and after purification

	Lys	Arg	Asp	Pro	Gly	Ala	Val	Leu	Phe
1. Resin [a]	2.89	8.97	0.90	N.D.	1.06	5.14	1.66	3.25	1.90
2. Purified [b]	2.76	6.21	1.04	1.94	1.05	1.01	1.06	2.99	1.96
3. Resin [a,c]	0.96	1.50	0.90	N.D.	1.06	5.14	1.66	1.08	0.95
4. Purified [b,c]	0.92	1.04	1.04	0.97	1.05	1.01	1.06	1.00	0.98

[a]Calculated from an average not including Arg, Ala, Val.

[b]Calculated from an average including all residues.

[c]Values in 1 and 2 divided by number of times that residue appears in peptide.

TABLE VI

Purity of peptides as demonstrated by amino acid analysis

Peptide	Amino Acid								
	Lys	Arg	Asp [Thr]	Pro	Gly	Ala	Val	Leu	Phe
I	0.92(3)	1.04(6)	1.04	0.97(2)	1.05	1.01	1.06	1.00(3)	0.98(2)
II	1.00(2)	1.02(6)	0.91	1.02(2)	1.01	1.01	1.01	0.98(2)	1.01
III	1.02(2)	1.03(5)	N.P.	1.01(2)	0.98	1.01	0.97	0.96	0.91
IV	0.95(2)	1.04(4)	N.P.	N.D.	0.96	1.07	0.98	1.00	0.95
V	1.00(2)	1.02(4)	N.P.	N.D.	0.93	N.P.	1.04	1.03	1.00
VI	0.96(2)	1.00(3)	N.P.	1.05(2)	1.05	N.P.	0.95	0.99	0.92
VII	0.97(2)	1.04(3)	N.P.	1.00	0.90	N.P.	N.P.	1.01	0.99
VIII	0.97(2)	1.00(4)	N.P.	1.02(2)	N.P.	N.P.	1.05	1.03	0.99
IX	0.94(2)	1.05(4)	N.P.	1.04(2)	N.P.	N.P.	0.98	0.97	0.97
X	0.96	1.02(4)	N.P.	N.D.	N.P.	N.P.	0.97	1.02	1.01
XI	1.02	1.01(4)	N.P.	N.D.	N.P.	N.P.	1.02	1.03	0.90
XII	0.94(2)	1.02(4)	N.P.	N.D.	N.P.	N.P.	0.96	1.09	0.93
XIII	0.96(2)	1.05(5)	N.P.	N.D.	N.P.	N.P.	0.95	0.98	0.91
XIV	1.00(2)	1.05(4)	N.P.	0.94	0.95	1.03	0.98	0.95(2)	0.96
XV	1.04(2)	1.03(5)	[0.96]	N.D.	1.02	1.04	0.92	0.96	0.96
XVI	1.00(2)	1.04(4)	[1.01]	N.D.	0.96	1.02	0.89	0.97(2)	0.96

All values are given as the amount of an amino acid divided by the number of times that amino acid is found in the peptide. The number in brackets is the number of residues of that amino acid found in the peptide where it exceeds one.

N.P. stands for amino acid not present; N.D. means amino acid not determined.

TABLE VII

History of Peptide XII

Synthesis	µmol/g resin	
Amino acid-Resin	98 (Arg)	
Peptide-Resin	78 (Phe)	Completion 79.6%
Cleavage	58 (Phe)	Cleaved 74.5%

Purification	µmol of peptide based on Val	
Peak 1	12	
Peak 2	28	
1 + 2	40	Pure peptide 70%
Sampled	6	
Total	46	
Total loaded	63	Recovery 73%
Overall yield	74.5 (70)(73) = 38.1%	

account the sample lost to the ninhydrin detection system and the amount of Val in the sample when it was loaded we have calculated that 73% of all the Val loaded onto the column was recovered. By multiplying % pure peptide by the % cleaved and the % recovery we have calculated that the overall yield for this peptide based on the amount of amino acid attached to the resin was 38%.

B. BIOLOGICAL CHARACTERIZATION

The obvious test of proper biological activity of the first peptide we synthesized was how well it duplicated the activity of Tn-I. Two properties characterize the activity of Tn-I. Firstly it inhibits actomyosin ATPase and secondly this inhibition is greatly enhanced by the presence of tropomyosin. It has been shown that this enhancement is closely related to F-actin binding of tropomyosin (Eaton et al., 1975). The cyanogen bromide fragment that peptide I was patterned after was also enhanced in its activity by the presence of Tm but no studies were carried out to determine whether Tm binding also increased.

1. Actomyosin ATPase Inhibition

The inhibitory activity of Tn-I, peptide I, and salmine in the presence and absence of Tm was assayed and the results are found in Fig. 13. It was clear that in the presence of Tm, peptide I inhibited the actomyosin ATPase but not as well as Tn-I. Tn-I inhibited approximately 50% of the full inhibition at a concentration of 0.8 nmol of protein/tube. At the same concentration of peptide I, the inhibition ranged from 30 to 36% of this maximum which suggests the peptide inhibited from 60 to 72% as well as Tn-I on a molar basis. This is within the range of 45 to 75% cited for the cyanogen bromide fragment (Syska et al., 1976).

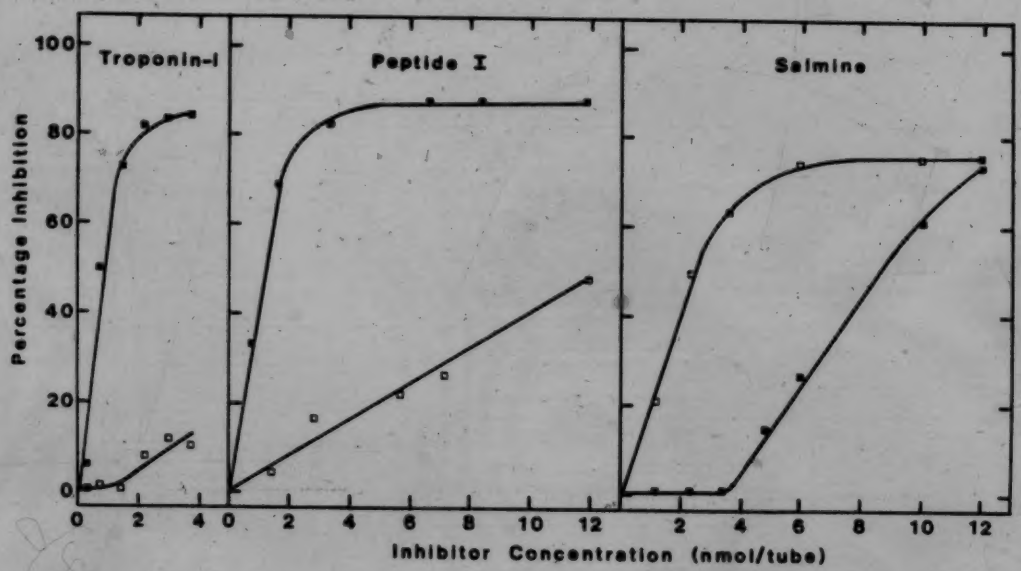


Fig. 13. Inhibition by rabbit skeletal Tn-I, peptide I and salmine of actomyosin ATPase. (■) 45 µg Tm/ml, (□) 0 µg Tm/ml. Zero percent inhibition was equal to the ATPase activity of actomyosin or if Tm is present it was the ATPase activity of actomyosin-tropomyosin. Conditions as in Procedure.

Both Tn-I and peptide I inhibit in the presence and absence of Tm but the inhibition is greatly increased in the presence of Tm. Many molecules have been shown to inhibit the actomyosin ATPase and the common feature they share is that they are basic proteins and polypeptides. In contrast to Tn-I and its cyanogen bromide fragment these basic proteins show no enhancement or a reduction in their inhibitory activity in the presence of Tm. Salmine (Fig. 13), an example of the latter case, highlights the fact that peptide I behaves as Tn-I does and thus its activity is due to specific interactions with actin and Tm rather than solely to its basic nature.

2. Enhancement of Tm Binding to F-actin

Since peptide I appeared to be a reasonable analog of the CNBr fragment we decided to use it to investigate whether the observed enhancement of activity in the presence of Tm was concomitant with an increase in binding of Tm to F-actin. These tests would have been difficult to do with the CnBr fragment because of the large amounts of peptide required. It has been shown that in 5 mM $MgCl_2$, 2 mM ATP and 30 mM KCl, conditions where a large part of the Mg^{2+} is not complexed by ATP, Tm both binds to F-actin and inhibits the actomyosin ATPase by itself and this inhibition can be increased by Tn-I (Eaton et al., 1975). The increase in inhibition did not have a corresponding increase in the amount of Tm bound. Fig. 14 demonstrates that under similar ionic conditions Tm gave 52% inhibition in the absence of inhibitor and that this rises to 85% upon addition of increasing amounts of peptide I. At 1 mM $MgCl_2$, 2 mM ATP and 30 mM KCl, conditions where much of the Mg^{2+} is complexed by ATP, it has been shown the Tm neither binds nor inhibits by itself but that both binding and inhibition are promoted by the addition of Tn-I (Eaton et al., 1975). Fig. 14 reveals that under similar ionic conditions Tm gives 14% inhibi-

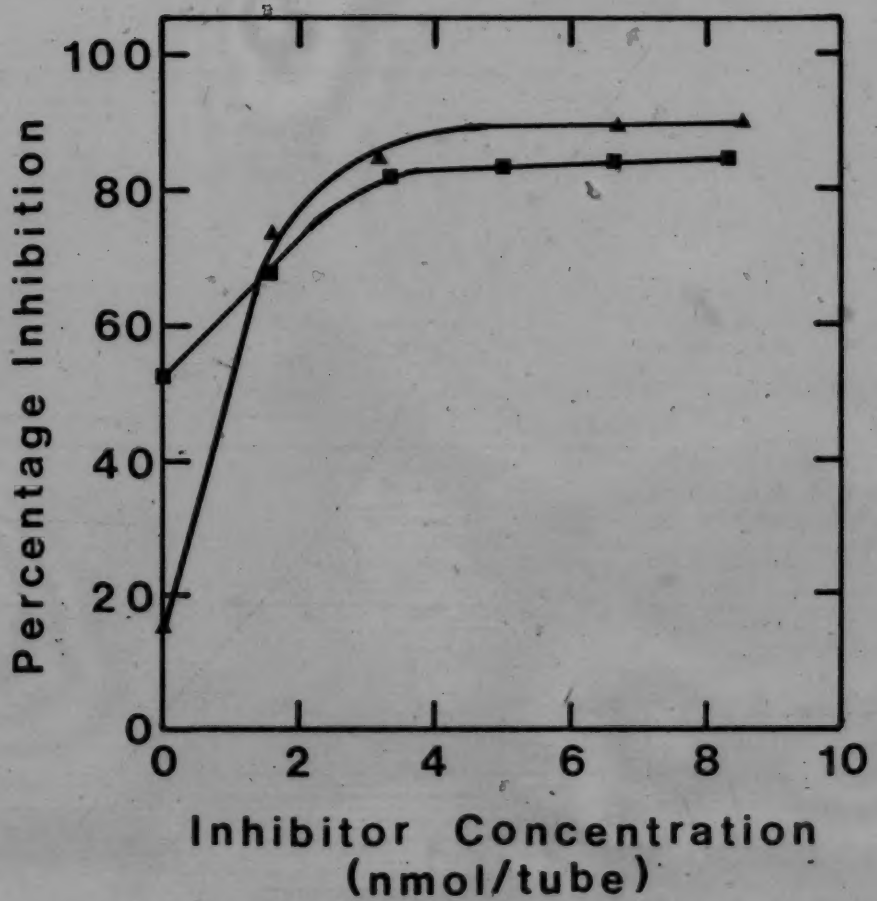


Fig. 14. Effect of ionic strength on peptide I inhibition of actomyosin ATPase. (▲) 2.5 mM $MgCl_2$ and 2.5 mM ATP; (■) 5.0 mM $MgCl_2$ and 2.5 mM ATP. Zero percent inhibition was equal to the ATPase activity of actomyosin. Other conditions as in Procedure.

tion by itself and that this can be increased to 90% by adding peptide I. Table VIII confirms that more Tm is associated with the F-actin pellet formed by centrifugation at 5.0 mM $MgCl_2$ and 2.5 mM ATP (high $[Mg^{2+}]$) than at 2.5 mM $MgCl_2$ and 2.5 mM ATP (low $[Mg^{2+}]$) and that the addition of peptide I (at low $[Mg^{2+}]$) also increased the amount of Tm associated with the F-actin pellet. These findings suggest that peptide I and by extension the CNBr fragment exert their inhibitory effect exactly as has been hypothesized for Tn-I. It is interesting that peptide I increased the inhibition of Tm even in the presence of millimolar free Mg^{2+} because under these conditions no further increase in the binding of Tm was found when Tn-I was added even though the ATPase inhibition did increase from 60 to 85% (Eaton et al., 1975). This was taken as evidence of a cooperative Tm-Tn-I effect in addition to the promotion of Tm binding to F-actin.

3. Summary

Peptide I inhibits actomyosin ATPase. This inhibition is greatly enhanced by the presence of Tm. Peptide I promotes the binding of Tm to F-actin and when no increase in Tm binding is possible peptide I cooperatively increases the inhibition of actomyosin ATPase by Tm. Therefore peptide I appears to have biological activity identical to the CNBr fragment of Tn-I and in addition both the CNBr fragment and peptide I are surprisingly good mimics of the activity of their parent protein.

4. Salmine Inhibition Continued

The salmine results are slightly complicated by our finding that on the basis of amino acid analysis it appears that our salmine was approximately 63% salmine AI and 37% salmine AII, which differs from AI by having one alanine, one isoleucine, no valine and perhaps one more arginine (Ando and Watanabe, 1969). With this caution in mind we have

TABLE VIII

Effect of ionic strength and peptide I on TM binding to F-actin

The conditions were 2.5 mM ATP, 20 mM KCl, 1 mM EGTA, 10 mM Tris-HCl (pH 7.6), 30 µg of actin/ml, 45 µg of tropomyosin/ml, in a 2-ml mixture. Pellets were formed by centrifuging at 25,000 rpm (37,000 x g) for 3 h. The amounts of each protein were found by scanning the gels (see Ch. 2 G).

Conditions	Tropomyosin bound to F-actin
	µg tropomyosin/µg F-actin
2.5 mM $MgCl_2$, 0 nmol peptide/tube	0.38
5.0 mM $MgCl_2$, 0 nmol peptide/tube	0.59
2.5 mM $MgCl_2$, 5.8 nmol peptide/tube	0.64

observed that the addition of salmine at high concentrations, to a solution containing actin or tropomyosin results in turbidity that can be removed by low speed centrifugation (1500 x g for 15 min). Gel electrophoresis of the resulting pellet indicates the presence of actin or tropomyosin. Examination of the salmine inhibitory curve (Fig. 13) shows that salmine does not begin to inhibit the actomyosin ATPase until approximately 4 nmol of salmine are present in the reaction mixture. Since 135 µg (2.04 nmol) of Tm are present this suggests that 1 mole of Tm can bind up to 2 moles of salmine. This may explain how Tm releases the inhibition caused by salmine. It is known that polylysine can cause actin filaments to cross-link (Griffith and Pollard, 1978). If the highly basic salmine affects actin similarly this would result in the inhibition of actomyosin ATPase. The addition of tropomyosin might result in the salmine preferentially binding to the tropomyosin rather than F-actin, thereby releasing the F-actin and restoring the actomyosin ATPase to its normal uninhibited values. That this competition is feasible is suggested by the fact that tropomyosin is an acidic protein containing 81 aspartate and glutamate residues, 53 arginine and lysine residues, and 2 histidine residues (Stone et al., 1974) while actin contains 51 aspartate and glutamate, 31 arginine and lysine, 8 histidine and 1 methyl-histidine residues (Elzinga et al., 1973). If this occurred the addition of less than 2 mole salmine for every mole of Tm, which results in no inhibition would give a pellet containing Tm but no actin. At molar ratios greater than 2 mole of salmine to 1 mole of Tm, we would expect to find increasing amounts with the Tm in the pellet. Table IX verifies that this predicted behaviour did occur.

TABLE IX

Effect of salmine on F-actin-tropomyosin pellet composition

Conditions were the same as for Table VIII except all mixtures were 2.5 mM $MgCl_2$ and the pellet was formed by centrifugation at 3700 rpm (1500 x g) for 15 min.

Molar ratio salmine/tropomyosin in mixture	Amount of protein in pellet	
	Actin	Tropomyosin
	μg	
1.5	0	33
2.4	12	45
9.1	51	56

CHAPTER IV

DELINEATION OF THE MINIMUM INHIBITORY REGION

After demonstrating the feasibility of solid-phase peptide synthesis of the inhibitory region of rabbit skeletal Tn-I it became possible to determine the actual inhibitory region. This would indicate the residues that were non-essential, make more economical the construction of analogs, and make comparison of Tn-I from various sources easier. One consideration, uppermost in our minds, during the synthesis of shorter analogs, was the possible complicating effect of the charges at the ends of these peptides. We decided that when we arrived at analogs that showed a reduction of activity we would construct analogs which had no terminal charges. In the case of the amino terminal it would be acetylated. This removes the amino terminal charge by formation of a peptide bond just as is found in the parent protein. We dealt with the problem at the carboxyl terminal by constructing the peptides as carboxyl terminal amides. This again removes the C-terminal charge on the α-carboxyl group by formation of the amide. Finally we tested the peptides in both acto-S-1 (A1) and actomyosin systems. The acto-S-1 (A1) system is a more facile one with which to work and remains active over a long enough period of time that all the assay results are from a single S-1 (A1) preparation.

A. SHORTENING FROM THE N-TERMINAL

The first three peptides we constructed (Peptides I, II, III, Table X) indicated that the sequence 98-103 was not important for inhibition. This indicated that even though the basic nature of the peptide

TABLE X

Effect of shortening the sequence from the NH_2-terminal.

Peptide No.	Sequence 98 … 104 … 110 … 117	Actomyosin I_{50}	n	S.D.	Acto-S-1(A1) I_{50}	n	S.D.
I	NH_2-Lys-Leu-Phe-Asp-Leu-Arg-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Arg-Val-Arg-Ala-OH	1.17	3	.14	1.00	3	.26
II	NH_2-Asp ——— Ala-OH	1.06	3	.25	1.09	2	.37
III	NH_2-Gly ——— Ala-OH	1.14	14	.28	1.07	2	.01

I_{50} is the nanomoles of peptide required to produce 50% of the total inhibition.

n indicates the number of inhibition curves, constructed from no less than 5 points each, from which the I_{50} was derived.

S.D. is the standard deviation.

is striking, deletion of Lys^{98} and Arg^{103} did not result in any loss of activity. The fact that the presence or absence of Asp^{101} in these peptides does not appear to make any difference also argues that if this portion of the sequence was important it would not be due to the basic groups alone since Asp^{101} would be expected to antagonize such an effect.

It is instructive to examine the other known sequences of Tn-I in this region. The sequences of rabbit, fast, slow and cardiac muscle are shown in Table II. Chicken fast is identical to rabbit fast in the region displayed on the table except that it contains Ser^{96} in place of Asn^{96}. The charged residues are conserved in all these sequences and the hydrophobes are in the main, conservatively replaced. In spite of this conservation it appears that the presence of these residues was not essential for activity.

No differences between actomyosin and acto-S-1 (A1) were found and the results shown in Table X verify this fact.

B. SHORTENING FROM THE C-TERMINAL

The first shortening we did of the C-terminal was not a straightforward removal of terminal residues. Peptide IV (Table XI) was identical to peptide III with the exception of a deletion of Arg^{114}. This deletion made peptide IV identical to the corrected sequence for rabbit skeletal fast Tn-I (Wilkinson and Grand, 1978) whereas peptides I, II and III were constructed according to the now believed to be incorrect original sequence (Syska et al., 1976). It was hoped that the activities of these two peptides would be different enough to allow us to test the correction in sequence. Their activities are too similar for any such test to be made (Table XI). All Tn-I molecules sequenced thus far delete Arg^{114}

TABLE XI

Effect of shortening the sequence from the COOH-terminal.

Peptide No.	Sequence 104 110 114 117	Actomyosin I_{50}	n	S.D.	Acto-S-1(A1) I_{50}	n	S.D.
III	NH_2-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Arg-Val-Arg-Ala-OH	1.14	14	.28	1.07	2	.01
IV	NH_2-Gly ——— Arg-Val-Arg-Ala- OH	1.18	2	.25	1.34	2	.30
V	NH_2-Gly ——— Arg-Val-Arg-NH_2	1.03	4	.10	1.13	5	.15
VI	NH_2-Gly ——— Arg-Val-NH_2	1.45	3	.22	1.08	2	.21
VII	NH_2-Gly ——— Arg-NH_2	2.50	3	.36	3.11	2	.05

except rabbit cardiac Tn-I (Table II). The importance of this exception is examined in Chapter V.

The fact that peptides III and IV appear to inhibit equally well was taken as evidence implying that residues 114 to 117 were not essentail to inhibiton. The reasoning for this was that if the peptide binds to exert its effect then the insertion of Arg in peptide III would result in the mismatching of residues 114 to 117 in the binding site. This mismatching would place an Arg at the position in the binding site where Val^{114} should be and similarly a Val and Arg at the rightful positions of Arg^{115} and Ala^{116} respectively. Since these can hardly be called conservative replacements this suggests that the sequence 114 to 117 may not be important.

To test this and to further refine the active site we constructed peptides V, VI and VII and compared their activities (Table XI). The proximity of so many basic residues to the C-terminal encouraged us to build these peptides as C-terminal amides. This ensured that interpreting the effect of shortening the peptide, ie. removing residues, would not be complicated by the effect of moving the negative charge closer to the inhibitory (positively charged) area. Comparison of peptides IV, V, VI (Table XI), revealed that the sequence 115 to 117 was not needed. Since the C-terminal residue of the cyanogen bromide fragment was Hse instead of the Met found in the protein and we had replaced it with Ala and still maintained good activity it was not surprising that this position could be omitted. The insignificance of Arg^{115} was very unexpected and reduced the potentially important basic residues to five from the original eight.

Equally unexpected was the discovery that the loss of Val^{114} caused a drastic drop in inhibitory activity. We had suggested that,

since peptides III and IV showed Val^{114} could be replaced with an Arg residue, the Val was not necessary. The demonstration of the importance of Val^{114} can be rationalized with the similar activities of peptides III and IV if Arg was a suitable replacement. Thus the requirement at position 114 would be for a bulky substituent. Another possibility was that the carbonyl of residue 114 or the α-amino group contributed by residue 115 must be present for high activity. Both these groups were present in peptides III, IV, V and VI (in VI the amino group of residue 115 was the amide of Val^{114}). Both these groups could participate in hydrogen bonds and thus aid the binding of this peptide. We have not distinguished between these two possibilities, namely the requirement for a bulky side chain at 114 or for the need of those groups involved in the peptide bond between residues 114 and 115. In this light it is interesting that all the Tn-I molecules sequenced thus far have either an Arg or Val at position 114 (Table II). Regardless, it is clear that either residue 114 or its α-carboxyl peptide bond but not the side chain of residue 115 was necessary for full activity. We felt therefore that this was the maximum shortening possible from the C-terminal.

Peptides III and IV (Table XI) also demonstrated that the distance between the negative charge of the α-carboxyl of the terminal amino acid and the inhibitory region does not automatically result in a difference in activity. Peptides IV and V (Table XI) reveal that the complete loss of this charge also resulted in no change. That our concern with the possible effects of this charge was justified was demonstrated by peptides IX and XII (Table XII). These peptides were identical except that peptide IX ends in an amide and hence has no negative charge on the α-carboxyl and showed a dramatic difference in activity. As ex-

TABLE XII

Effect of the COOH-terminal charge.

Peptide No.	Sequence	Actomyosin			Acto-S-1(A1)		
		I_{50}	n	S.D.	I_{50}	n	S.D.
IX	NH_2-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-NH_2	2.46	4	.17	1.88	3	.19
XII	NH_2-Lys ——— Arg-OH	3.45	2	.21	3.38	2	.10

pected the presence of a negative charge so close to the positively charged inhibitory region resulted in a loss of activity. In summary then, it appears that the charge at the end of a peptide may have no effect on activity when it is far enough from the active region of the peptide but this can change if it is moved closer.

Once again, there was no significant difference between the inhibitory figures derived from acto-S-1 (A1) or actomyosin assays.

C. FURTHER SHORTENING FROM THE N-TERMINAL

While further shortening this sequence from the amino terminal we took the precaution of acetylating the amino terminal of selected peptides to enable us to discount the effect of the charge on the α-amino group. Comparison of peptides V and VIII (Table XIII) showed that the positively charged α-amino group of Gly^{104} contributed in a small way if at all to the inhibition. This precise assignment could be made because the acetyl group could be regarded as an analog of glycine where the amino group of glycine has been replaced by a hydrogen. While peptides VIII and X (Table XIII) suggest that the Lys^{105} side chain was essential to inhibition the possibility that the groups involved in the peptide bond between residues 104 and 105 or the α carbon and two hydrogens of residue 104 were essential, have not been excluded. The latter at least seems unlikely enough to be discounted completely.

Peptides VIII and IX (Table XIII) suggest either that the presence of a positive charge on the α-amino group of residue 105 or the loss of the groups involved in the peptide bond resulted in a substantial loss of inhibitory activity. If it was the former the loss of activity could be explained by competition between the α and ε-amino group of Lys^{105} for

TABLE XIII

Further shortening of the sequence from the NH_2-terminal.

Peptide No.	Sequence 104	110	115	Actomyosin I_{50}	n	S.D.	Acto-S-1(A1) I_{50}	n	S.D.
V	NH_2-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-NH_2			1.03	4	.10	1.13	5	.15
VIII	Ac-Lys	_____	Arg-NH_2	1.36	3	.05	1.28	2	.11
IX	NH_2-Lys	_____	Arg-NH_2	2.46	4	.17	1.88	3	.19
X	Ac-Phe-	_____	Arg-NH_2	2.85	2	.07	2.73	3	.05
XI	NH_2-Phe-	_____	Arg-NH_2	3.12	3	.10	3.30	2	.21

ion pair formation with a negatively charged group at the binding site. Such a competition might result in less favorable interactions for the other residues on the peptide involved in binding and hence lower activity. If the loss of the groups involved in the peptide bond was responsible for the lower activity then the loss of hydrogen bonds these groups may be involved in would explain the lowered activity. In contrast peptides X and XI (Table XIII) show that neither the positive charge on the α-amino group of Phe^{106} or the groups involved in the peptide bond between residues 105 and 106 had much effect on inhibitory activity.

These studies indicate that the side chains of all the amino acids up to and including Gly^{104} are not essential to inhibition. Furthermore, with the α-carboxyl charge effects discussed earlier, they have proven our contention that great care must be taken in assigning the presence or absence of activity in a protein fragment to the sequence of the fragment without taking into account the charged groups that exist at either end of peptide. These charges obviously do not exist in a protein unless the fragment contains either the amino or carboxyl terminus of the protein and even then only one charge would be legitimately present in the peptide. This caveat applies equally to peptides generated by synthetic or natural (e.g. proteolytic cleavage) methods and therefore it seems to us that all peptides with sequences identical to a region of the parent protein should be considered analogs unless the terminal charges are masked.

D. SUMMARY

We have summarized the residues whose side chains were not important to inhibition in Fig. 15. This shows that the region of the peptide (residues 105-114) whose side chains may be important to inhibition in-

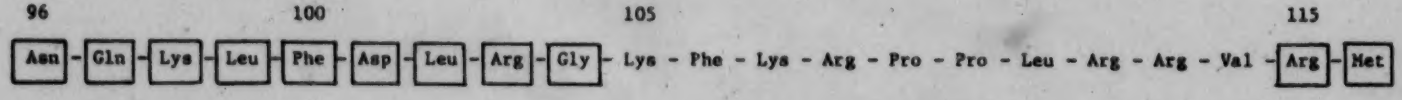


Fig. 15. Non-essential amino acids of the inhibitory sequence of rabbit fast Tn-I. (□) non-essential amino acids.

cludes five basic residues, three hydrophobes and two prolines. Of these the side chains of Lys^{105} and Val^{114} may be tentatively assigned as essential. Additional evidence that our assignment of non-essential residues was correct appeared in a recently published paper (Nazaki et al., 1980). These researchers demonstrated that a peptide corresponding to the region 101 to 115 was active whereas fragments corresponding to 101 to 109 and 110 to 115 were not. This is entirely consistent with our delineation of the minimum inhibitory region as 105 to 114.

We have found no significant differences between the acto S-1(A1) and the actomyosin systems. Therefore we used only the acto-S-1 (A1) system to examine the inhibition of skeletal Tn-I, a representative peptide of high activity (peptide V) and a representative peptide of low activity (peptide XI). Peptide XI differed from peptide V by lacking Gly^{104} and Lys^{105} (Table II). While the peptides with good solubility were easily assayed, Tn-I with its notorious solubility problems required a good deal of ingenuity (see Chapter II) to assay. As a result of this we rarely achieved full inhibition at ratios of Tn-I to actin reported in the literature e.g. 0.3 to 1 (Eisenberg and Kielley, 1974). Even after we appeared to have the insolubility beaten we found we still could not achieve this ratio. The most significant difference between our assay conditions and those of Eisenberg was our substantially lower actin concentration. Fig. 16 also investigates the effect then of low and high actin concentrations on rabbit skeletal Tn-I and our representative peptides.

The curves for Tn-I indicated that actin concentration did effect the efficiency of inhibition. At low actin concentration (open circles) maximum inhibition by Tn-I occurs at a ratio of inhibitor to actin of

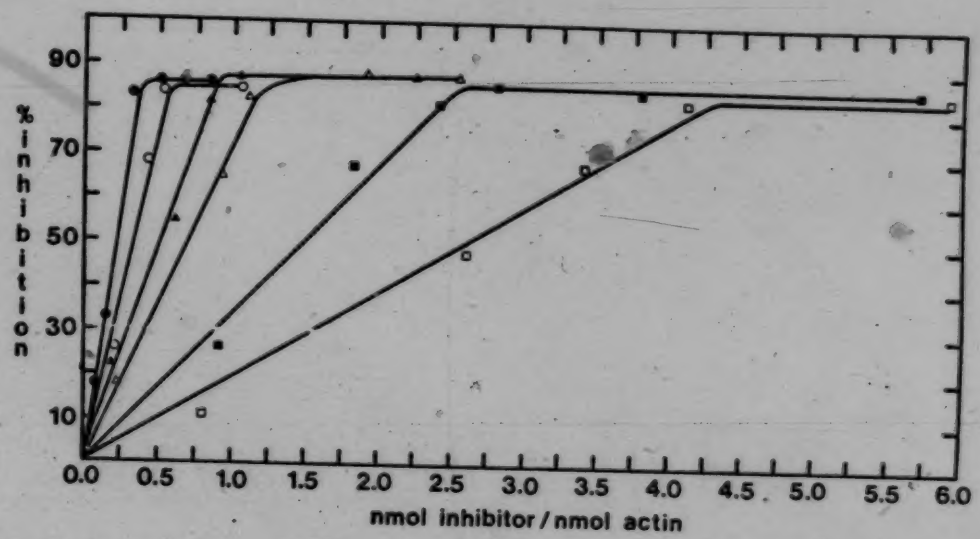


Fig. 16. Effect of actin concentration on inhibition. (●,○) fast Tn-I; (▲,△) peptide V; (■,□) peptide XI; (●,▲,■) assays contained 473 µg, Tm, 315 µg actin, 90 µg S-1 (A1); (○,△,□) assays contained 135 µg Tm, 90 µg actin, 90 µg S-1 (A1). Other conditions as in Procedure.

greater than 0.5 to 1. At high actin concentration (closed circles) maximum inhibition occurs at a ratio of about 0.3 to 1. Similarly the efficiency of both peptides was increased at the high actin concentration (closed symbols compared to low actin concentration (open symbols). One possible explanation for this is that at lower actin concentrations proportionately more rigor complex (Bremel et al., 1972; Bremel and Weber, 1972) was formed and the rigor complex antagonizes the inhibition of both Tn-I and the peptides.

Fig. 16 gave a far more graphic description of the differences in activity between an active peptide (peptide V) and one lacking an essential residue (peptide XI) that the I_{50} values do. Another way of illustrating this was to examine the percentage of full inhibition that each peptide exhibited at a ratio of inhibitor to actin that resulted in 50% of the full inhibition with rabbit skeletal fast Tn-I. From Fig. 16 we found that at the low actin concentration the figures were 22.4% and 6.7% and at the high actin concentration the figures were 23.1% and 7.4% for peptide V and XI respectively. Therefore the active peptide could be said to demonstrate approximately 45% of the activity of Tn-I. The corresponding figure for the inactive peptide was 14%.

In conclusion then we feel that the high activity of peptides containing the sequence 105 to 114 proved that these peptides were excellent mimics of rabbit skeletal Tn-I, a protein at least 17 times larger. In addition given the peptides high activity, taken together with their previously demonstrated enhancement of this inhibition by tropomyosin, the inhibition they can cause in addition to that enhanced by tropomyosin and finally the similarity of their response to increased actin concentration to the response of Tn-I, all make us reasonably confident that this region is of great significance to the function of Tn-I.

CHAPTER V

COMPARISON OF THE INHIBITORY SEQUENCE OF VARIOUS SPECIES OF Tn-I

"The treasures of the house do not come in by the front door" [6]

A. Tn-I ACTIVITIES IN A RABBIT FAST ACTOMYOSIN SYSTEM

Since we had established the significance of the region 105 to 114 to the inhibition caused by rabbit skeletal Tn-I we decided to attempt to relate differences in the inhibitory ability of various species of Tn-I to differences in their sequences in regions homologous to this region. The sequences of four species of Tn-I were known when we embarked on this study; chicken fast, rabbit fast, rabbit slow and rabbit cardiac (Wilkinson and Grand, 1978). The sequences homologous to 105 to 114 in rabbit skeletal Tn-I are shown in Fig. 17.

1. Comparison of Chicken Fast, Rabbit Fast and Rabbit Slow Muscle Tn-I

The sequences of the inhibitory region of chicken fast, rabbit fast and rabbit slow muscle Tn-I were identical. It was known that rabbit slow muscle Tn-I inhibits in rabbit fast actomyosin systems poorly compared to rabbit fast muscle Tn-I (Syska et al., 1974). These two facts, identical sequence, different activities, suggested that even though the inhibitory region 105 to 114 was present in both proteins, its presence alone was not enough to ensure good inhibitory activity and therefore other portions of the protein could modify the activity of this region in Tn-I. This interpretation was supported by the fact that a peptide generated from rabbit skeletal Tn-I containing the sequence 64 to 133 exhibited less activity on a molar basis than the cyanogen bromide frag-

[6] Master Mumon (in: The Wisdom of the Zen Masters, I. Schloegl, Sheldon Press, U.K.) p. 48.

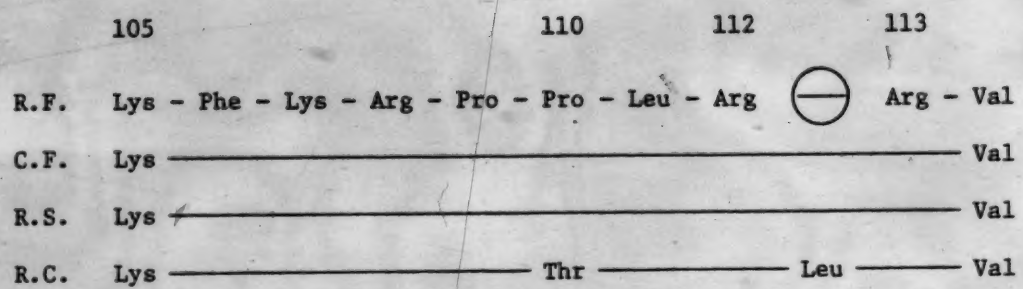


Fig. 17. Inhibitory region sequence of chicken fast; rabbit fast, slow and cardiac Tn-I. Chicken fast (C.F.), Rabbit fast (R.F.), Rabbit slow (R.S.) and Rabbit cardiac (R.C.) (⊖) indicates residue deleted.

ment 96 to 117 (Syska et al., 1976). These authors suggested that the larger peptide adopted a different conformation from that of the intact molecule which renders the inhibitory region less active. The crudely defined inhibitory region of these authors (96-117) contained five places where the sequences differ from rabbit fast to slow Tn-I. Since any one or combination of these five changes could have been responsible for the reduced activity of rabbit slow Tn-I relative to fast Tn-I it was impossible to unequivocally state that changes remote from the inhibitory region were lessening the activity of rabbit slow muscle Tn-I. Since our narrowly defined inhibitory site contains no amino acid changes, we conclude portions other than the inhibitory region of Tn-I 105-114 can modify the activity of the inhibitory region and as a result affect the activity of the whole molecule. Since the activities of rabbit slow muscle Tn-I and the large rabbit fast muscle Tn-I fragment 64-133 were lower than rabbit fast Tn-I and the small rabbit fast muscle Tn-I fragment (96-117) respectively, we know this modification can take the form of a reduction in activity. In addition we know that the inhibitory region can be enhanced in activity by other portions of the Tn-I molecule because as demonstrated in the previous chapter and by Syska et al., (1976) peptides containing the inhibitory region inhibit less well than rabbit fast Tn-I.

2. Comparison of Rabbit Fast and Rabbit Cardiac Tn-I

A comparison of rabbit fast and rabbit cardiac muscle sequences Fig. 17 revealed that there were two amino acid changes in the inhibitory region. These were the substitution of Pro^{110} by Thr and the insertion of a Leu residue between Arg^{112} and Arg^{113} (Fig. 17). Neither of these changes was conservative.

The substitution of Pro^{110} by Thr results in the replacement of a non-polar amino acid by a polar one. In addition the peculiar geometry

of proline which could be important to the conformation of the molecule would be lost. In particular, proline has been implicated as a very important contributor to β turns especially at position 2 and its substitution at this position by Thr reduces the β turn forming potential by 65% (Chou and Fasman, 1974). Either or both of these effects might result in a change of activity.

The insertion of a Leu between Arg^{112} and Arg^{113} could also affect the activity of Tn-I in one of two possible ways. The introduction of the hydrophobic Leu for the charged Arg^{113} might have changed the activity. Another possibility was that the resulting mismatch of the residues from 113 on, could result in a change of activity. We found in the preceding chapter that this latter possibility was not important.

Therefore there were potentially significant differences in the inhibitory region 105-114. Were these reflected in different activities in their parent proteins? It has been demonstrated that rabbit cardiac Tn-I inhibits considerably less well than rabbit fast Tn-I (Syska et al., 1974) in a rabbit fast actomyosin system. We duplicated these results as shown in Figure 18. The construction of peptide analogs was initiated to determine which of the two amino acid changes were significant if any, and if possible to explain why they were significant.

Peptides III, IV and XIV were constructed and assayed in a rabbit fast muscle acto-S-1 (A1) system (Table XIV). Peptides III and IV once again affirmed that the mismatch of residues did not result in a loss of activity. The peptide results in Table XIV were derived from the same S-1 preparation but differ slightly from those given in Chapter IV because a different S-1 preparation was used. Peptide XIV dem-

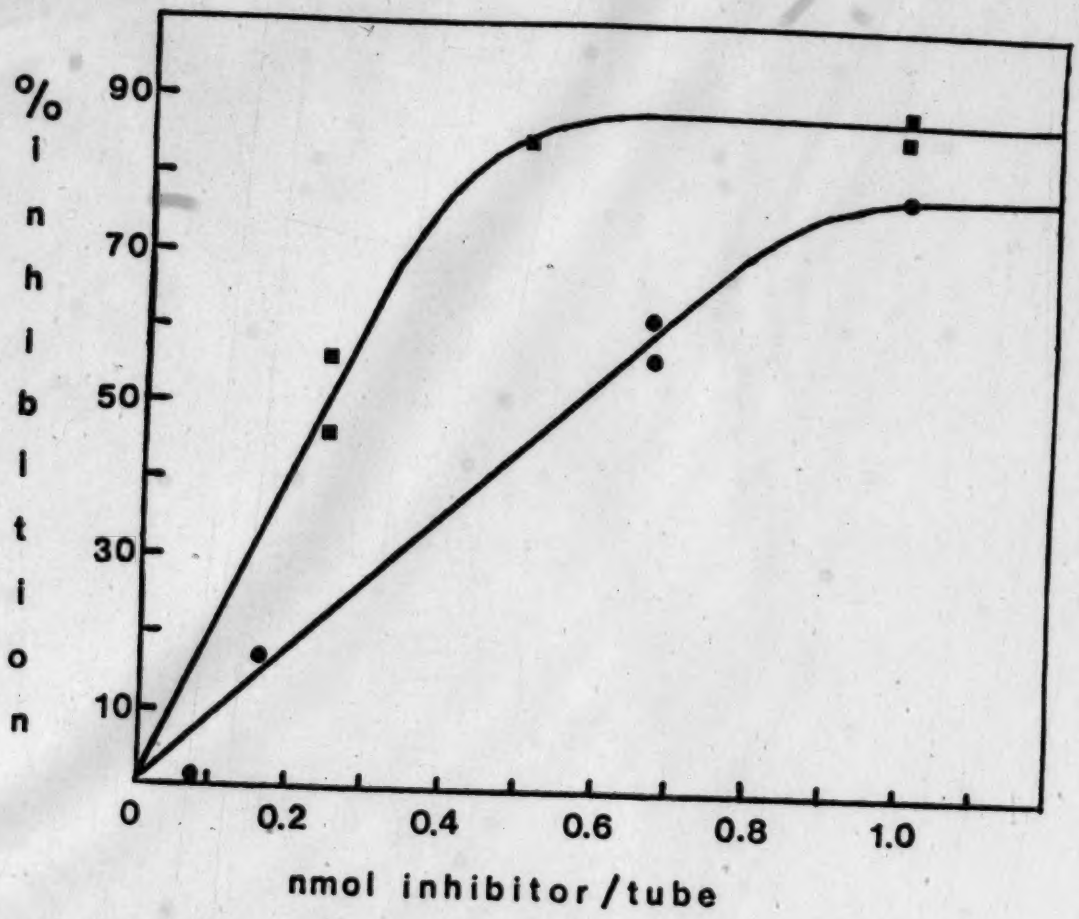


Fig. 18. Inhibition by rabbit cardiac and fast Tn-I of rabbit skeletal actomyosin ATPase (●) cardiac Tn-I, (■) fast Tn-I. Other conditions as in Procedure.

onstrated quite convincingly that the replacement of the arginine residue by leucine resulted in a large decrease in activity. We concluded that the major effect of this change was to decrease activity probably as a result of the loss of the charged side chain of arginine, rather than the introduction of the bulky side chain of leucine. We came to this conclusion since, if anything, arginine has a bulkier side chain than leucine.

Peptides XV and XVI were also constructed and assayed in a rabbit fast muscle acto-S-1 (A1) system (Table XIV) to test the effect of the threonine substitution. Comparison of peptides III and XV reveal that the Thr substitution has no effect on activity.

Our conclusion was that the replacement of arginine with the concomitant loss of the positive charge on its side chain was solely responsible for the drop in activity of peptide XVI relative to peptide IV. Since peptide XVI was an analog of the rabbit cardiac Tn-I and peptide IV was an analog of the rabbit fast Tn-I we feel that this insertion contributes in a major way to the reduced activity of rabbit cardiac Tn-I in the rabbit fast actomyosin system.

B. Tn-I ACTIVITIES IN CARDIAC AND HYBRID ACTOMYOSIN SYSTEMS

1. Hybrid and Bovine Cardiac Actomyosin Assays

The activities of various species of Tn-I have been tested, if at all, mainly in rabbit fast actomyosin systems. The only report of them being tested in another system, used bovine cardiac muscle as the actomyosin source (Hincke et al., 1977). Interestingly, these assays showed that although bovine cardiac Tn-I inhibited more poorly than rabbit fast Tn-I in a rabbit fast actomyosin system, it inhibited

TABLE XIV

Inhibition by rabbit fast and rabbit cardiac peptide analogs of Tn-I in rabbit fast acto-S-1(A1) ATPase assays

Peptide No.	Sequence 104	110	112	113	116	I_{50}	n	S.D.
III	Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Arg-Val-Arg-Ala					1.21	3	.04
IV	Gly ——— ⊖ ———				Ala	1.48	3	.32
XIV	Gly ———		Leu	———	Ala	3.30	2	.42
XV	Gly ———	Thr	———		Ala	1.26	2	.25
XVI	Gly ———	Thr	———	Leu ———	Ala	3.45	2	.42

⊖ indicates residue deletion.

better than rabbit fast Tn-I in a bovine cardiac actomyosin system. These findings led to the suggestion that a Tn-I inhibits most actively in its "parent" actomyosin system.

The relative reversal in activities of the Tn-I species between the two systems led to some speculation about the specificity of the inhibition. We have duplicated these results using systems that consisted of; rabbit α-Tm, rabbit skeletal actin or bovine cardiac actin and rabbit skeletal myosin or bovine cardiac myosin. The source of the actin made no difference, not surprisingly, given the highly conserved nature of actin; bovine cardiac and skeletal actins differ by only four amino acids (Vandekerckhove and Weber, 1978). The source of the myosin determined which Tn-I would be the better inhibitor (Fig. 19). It was difficult to reconcile this result with the steric blocking model of inhibition. If the actin was blocked more completely by fast Tn-I than cardiac Tn-I it was not easy to see how merely changing the source of myosin could reverse the inhibition patterns of the Tn-I. A further possibility exposed by these studies was that even in thin filament regulation the nature of the myosin must be considered.

2. Hybrid Rabbit Actomyosin Assays

Interesting as the above results were, they were pursued no further because no sequence for bovine cardiac Tn-I existed and we were forced to use rabbit cardiac actomyosin because the sequence of rabbit cardiac Tn-I was known. The question we wanted to examine was whether our peptide analogs behaved similarly to their parent proteins. In other words if the rabbit cardiac system reacted like the bovine cardiac system then the analogs would inhibit best in their own system.

The assay systems consisted of myosin or S-1, actin and tropo-

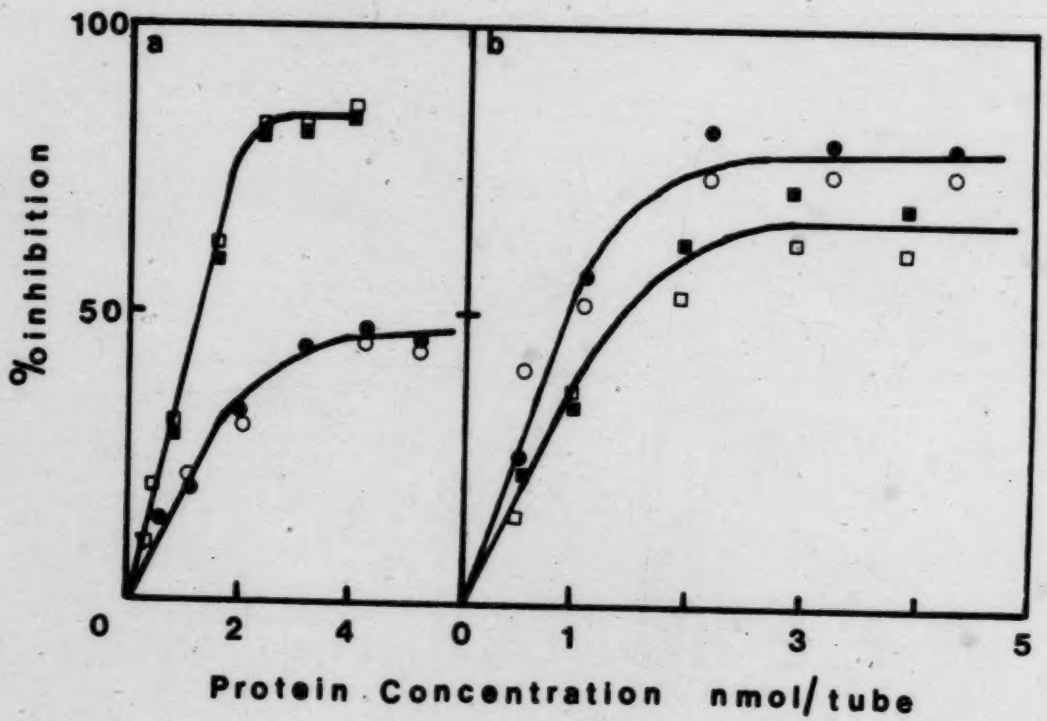


Fig. 19. Relative activities of rabbit fast and bovine cardiac Tn-I in rabbit skeletal and bovine cardiac actomyosin ATPase assays.
(a) Cardiac or skeletal actin; skeletal myosin. (●,■) cardiac actin in assay, (○,□) skeletal actin in assay, (●,○) cardiac Tn-I, (■,□) fast Tn-I.
(b) Cardiac or skeletal actin; cardiac myosin. (●,■) skeletal actin in assay, (○,□) cardiac actin in assay, (●,□) cardiac Tn-I, (■,□) fast Tn-I.

myosin. In either the rabbit cardiac or rabbit fast skeletal assay system, the tropomyosin was cardiac Tm. It has been established that rabbit cardiac and rabbit skeletal α-Tm were identical (Lewis and Smillie, 1980) and α-Tm accounted for 77% of skeletal muscle tropomyosin (Cummins and Perry, 1973) so keeping the source of the tropomyosin constant seemed justified. Actin from both rabbit cardiac and rabbit skeletal muscle was used but as no significant differences were ever found, all the assays reported, except the first, contain the combined results calculated by averaging the values for both actins.

a. Peptide inhibition

To illustrate exactly what was meant by no significant differences in assay results regardless of the actin source, Table XV contains the results of the fast skeletal and cardiac Tn-I analogs tested in assay systems constructed with fast skeletal S-1 and either skeletal or cardiac actin. The combined results emphasize the similarities. It was also obvious that once again the insertion of Leu was solely responsible for the reduction in activity of the cardiac peptide analogs (XIV, XVI) in an assay system that included rabbit fast skeletal S-1.

When the analogs were tested in an assay system that included rabbit cardiac myosin it was found that the cardiac analogs did not inhibit as well as the fast skeletal analogs (Table XVI). In fact they inhibited more poorly in the cardiac system than they had in the fast system (Table XV). The insertion of the Leu was once more solely responsible for the drop in activity.

b. Tn-I inhibition

These results could be explained in one of two ways. First, the cardiac systems of rabbits and cows are not analogous. If this

TABLE XV

Activities of rabbit fast and cardiac Tn-I analogs in hybrid rabbit acto-S-1(Al) ATPase assays: skeletal or cardiac actin, fast S-1(Al)

Peptide No.	Fast actin fast S-1(Al)			Cardiac actin fast S-1(Al)			Combined		
	I_{50}	n	S.D.	I_{50}	n	S.D.	I_{50}	n	S.D.
III	1.21	3	.04	1.31	2	.04	1.25	5	.06
IV	1.48	3	.32	1.53	2	.05	1.50	5	.20
XIV	3.30	2	.42	3.32	2	.51	3.31	4	.38
XV	1.26	2	.25	1.35	2	.11	1.30	4	.30
XVI	3.45	2	.42	3.27	2	.25	3.36	4	.30

TABLE XVI

Activities of rabbit fast and cardiac analogs in hybrid rabbit actomyosin ATPase assays: skeletal or cardiac actin, cardiac myosin.

Peptide No.	Fast and cardiac actin, cardiac myosin		
	I_{50}	n	S.D.
III	1.28	4	.15
IV	1.48	3	.09
XIV	5.45	3	.74
XV	1.34	4	.25
XVI	5.95	3	.73

were the case the skeletal Tn-I might inhibit better than the cardiac Tn-I regardless of which system was used and the peptides reflected this situation. In support of this postulated difference in the regulation of cow and rabbit hearts was the finding that β Tm is present to about 20% of the total in large and slowly beating hearts (sheep, pig, human) but absent in smaller rapidly beating hearts (rabbit, guinea pig, dog, Leger et al., 1976). The second possible explanation was that the reversal did occur with whole Tn-I molecules but not with the inhibitory region peptides and that this reversal was somehow achieved by portions of the Tn-I molecule other than the inhibitory region.

To distinguish between these possibilities we assayed the activities of rabbit cardiac and fast Tn-I in assay systems containing either skeletal or cardiac myosin. The purity of the Tn-I used is shown in gels in Fig. 20. The I_{50} values for the Tn-I species in the two systems is shown in Table XVII and representative cardiac actomyosin inhibition curves are found in Fig. 21. These prove very convincingly that the peptide analogs behave exactly as do the parent proteins and so it was not necessary to invoke the involvement of other portions of the Tn-I molecule in the inhibition. These results also suggest that the inhibitory region of bovine cardiac Tn-I should be different from rabbit cardiac and fast Tn-I since it behaved in a different manner than either of these, i.e. inhibits best when bovine cardiac myosin was present.

C. SUMMARY

Taking all the evidence presented in the previous chapters we have the scaffolding to support more extensive investigations of the

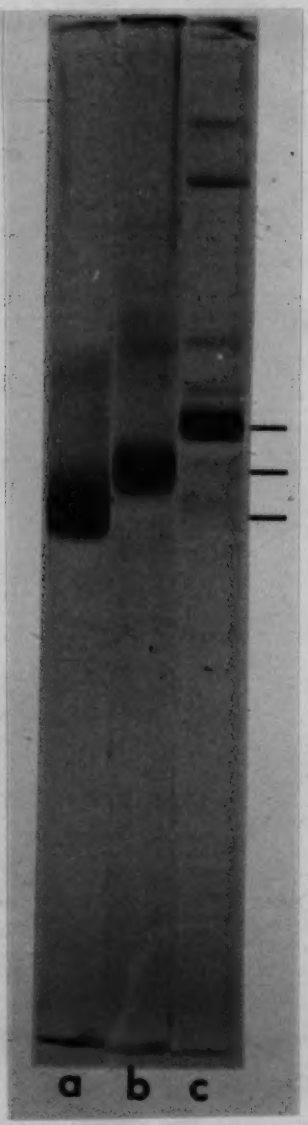


Fig. 20. Gel electrophoresis of rabbit cardiac, fast Tn-I and fast Tn-C.
(——) marks position of protein.
(a) 40 µg fast Tn-C.
(b) 40 µg fast Tn-I.
(c) 40 µg cardiac Tn-I.

TABLE XVII

Activities of rabbit fast and cardiac Tn-T in hybrid rabbit actomyosin ATPase assays: skeletal or cardiac actin, skeletal or cardiac myosin

Tn-I	I_{50}	n	S.D.
	Cardiac or skeletal actin: skeletal myosin		
Fast	.32	2	.001
Cardiac	.70	2	.003
	Cardiac or skeletal actin: cardiac myosin		
Fast	.17	2	.05
Cardiac	.56	4	.05

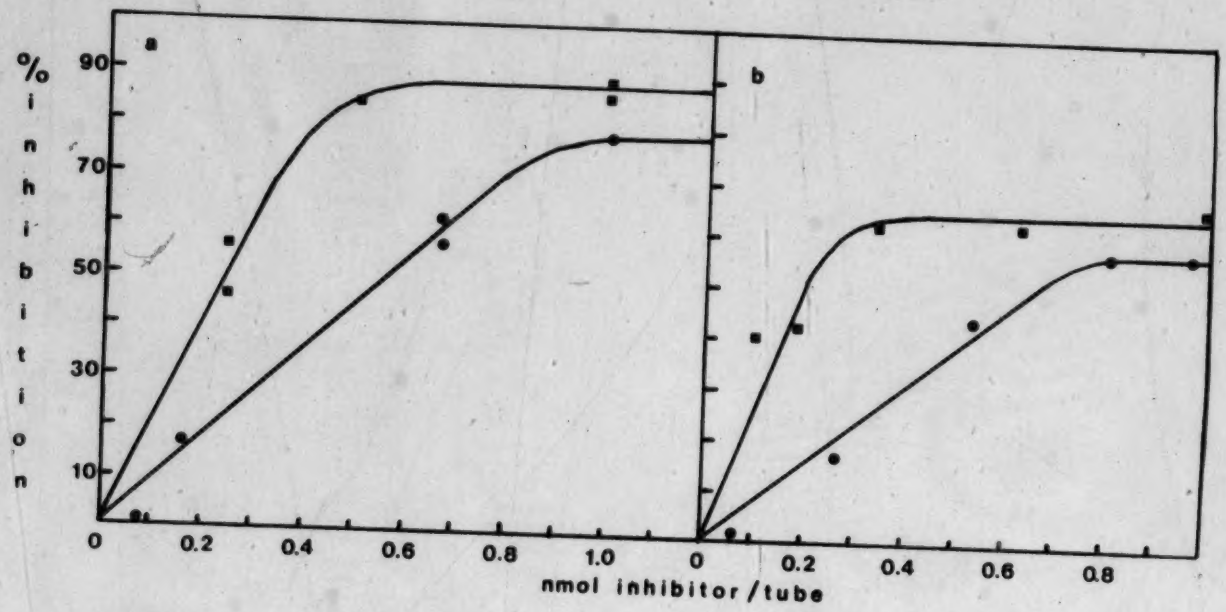


Fig. 21. Inhibition by rabbit cardiac and fast Tn-I of hybrid rabbit actomyosin ATPase: Skeletal or cardiac actin, skeletal and cardiac myosin.
(a) Skeletal or cardiac actin, skeletal myosin.
(b) Skeletal or cardiac actin, cardiac myosin. (■) fast Tn-I, (●) cardiac Tn-I. Other conditions as in Procedure.

inhibitory region of Tn-I. In addition to establishing the minimum size of this region we have determined that in the interior of this region at least one residue has a side chain that was unimportant (position 110) and one residue whose positively charged side chain was crucial (Arg 113) to high inhibitory activity in a rabbit skeletal actomyosin system (Fig. 22). It also would seem that the replacement of Arg^{113} by Leu in cardiac Tn-I was responsible for some of the reduced activity of cardiac Tn-I relative to fast Tn-I in both systems.

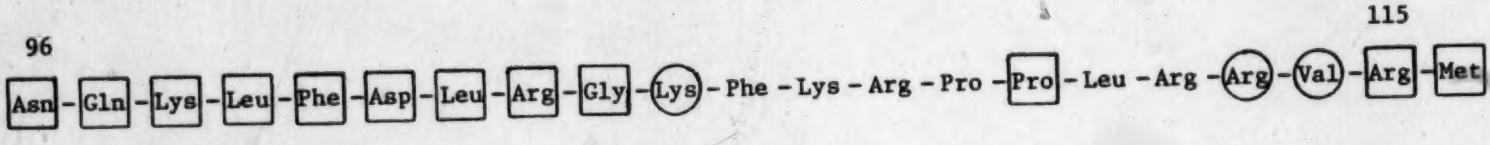


Fig. 22. Relation of CNBr inhibitory fragment sequence to biological function. (□) amino acids shown to be non-essential for inhibition by this study, (○) amino acids shown to be essential for inhibition by this study.

CHAPTER VI

CODA

> "Do you know why you cannot wait for the shot and why you get out of breath before it comes? The right shot at the right moment does not come because you do not let go of yourself. You do not wait for fulfillment, but brace yourself for failure."[7]

A. FURTHER INVESTIGATIONS

The advantages of solid phase peptide synthesis over other methods that use the parent protein as raw material have been highlighted in this study. It has often been mentioned that synthesis allows the generation of sequences that do not exist in nature. In this case it is particularly true because with the exception of fast Tn-I there was no other way to generate a short peptide sequence containing the inhibitory site. Another neglected advantage was that the peptides with their good solubility and relatively easy purification were far more tractable than Tn-I. Another strong point was the precise and unambiguous assignment of important residues that synthesis makes possible compared to modification of proteins or protein fragments. These advantages along with our findings lead us to believe that further investigations might prove fruitful. A few of the areas we plan to investigate in the future follow. These studies we hope will aid us in clarifying some questions about muscle interactions or protein interactions or both.

1. Construction of More Analogs

Although we have established the potential importance of three of the ten residues at the inhibitory site there are still seven whose contributions we do not know. In particular it would be interesting to

[7] Zen in the Art of Archery. E. Herrigel, Random House, New York pg 33.

determine if any of the other basic residues can be deleted without affecting the activity. The easiest way to do this would be to replace each of these in turn with a glycine and examine their activity. By this method also it would be possible to measure the contributions of the remaining hydrophobes and proline. At the end of these studies it should be possible to construct the simplest inhibitory site. It would consist of nothing but required residues and glycine as space fillers. This would give us some information about what the complementary binding site looks like. This view of the binding site would not only tell us what groups would be there but because of the steric constraints imposed by the structure of the peptide some geometric arrangements of those groups in the complementary binding site could be eliminated as unlikely to occur.

2. Location of the Complementary Binding Site

Direct understanding of the complementary binding site is also possible through some of the work we are undertaking on cross-linkers. It appears possible to synthesize peptides containing the inhibitory region and chemical or photochemical cross-linkers. Properly manipulated these could reveal the interaction site on both a molecular and residue level. For the former it should be possible to show whether the peptide interacts with actin or a site jointly made of actin and tropomyosin. On the basis of other less precisely located cross-linkers (Sutoh, 1980) we would expect only actin to cross-link suggesting an allosteric mechanism for Tn-I promotion of Tm to F-actin binding and Tm inhibition of actomyosin ATPase. At the level of the residues involved it should be possible to cross-link radioactive peptide to actin-Tm and after digestion locate the actual sequence to which it

binds. This effort would locate the residues postulated to exist in the complementary site by the analog studies. Varying the location of the cross-linker within the inhibitory sequence and allowing these peptides to cross-link would allow us to locate other sequences that may contribute to the complementary site. By sequencing the peptide and locating the cross-link it may even be possible to assign the binding of that particular part of the inhibitory region to a specific sequence of the complementary binding site. This kind of information coupled with that described in the previous section would allow us to construct a model that would give a three dimensional picture of the entire inhibitor interaction site.

It may also prove possible to cross-link under other physiologically important conditions, e.g. ± Tn-C and Tn-T then ± Ca^{+2}, to determine if changes in the location of the cross-links may give clues to the conformational changes occurring.

3. Studies on Binding and Conformation

It should prove possible with the introduction of NMR reporter groups like fluorophenylalanine or radioactive groups such as C^{14} glycine to measure the strength of peptide binding to actin or actin-Tm. This will undoubtedly be complicated by the peptide promotion of binding of Tm to actin but should still provide precise information to answer the question of whether binding and inhibition are related or if, in fact, a peptide can bind without inhibiting.

Another area it seems to us suitable for examination is whether secondary structure exists in the peptide itself and if not, whether structure or at least a conformational change is imposed on it during binding. These studies could be most easily carried out once

again with NMR. Recent developments with laser CIDNP experiments indicate that NMR may also be exploited to give information on which portions of inhibitory peptides are no longer exposed to the aqueous media when the peptides bind or alternatively the measurements could show that a portion or all of the peptide remains accessible even though bound.

B. REFLECTIONS ON THE FREQUENCY OF ACTIVE REGIONS THAT ARE SHORT PEPTIDE SEQUENCES

One point that has perplexed us ever since we embarked on the studies was amazement that a peptide of such small size could duplicate so many of the actions of its parent protein so well. As the evidence for this grew, so did our amazement. Now, in defence of the rest of the protein, it is obvious that many of the required properties of Tn-I, e.g. interactions with Tn-T and Tn-C are probably located in the rest of the molecule but the inevitable suspicion of such good luck lingers. If we were not lucky, what explanation was there for the rarity of active regions, e.g. protein-protein interaction sites, protein-small molecule (peptide hormones, ions, etc.) interaction sites, that are only short peptide sequences? After pondering this question for some time we think that the example our inhibitory peptides set, shed some light on the question. In so doing the peptides provoke some important questions of their own.

To find why these type of "linear" active regions are rare it might be useful to consider the two methods by which active regions are discovered, testing proteolytic cleavage fragments and X-ray crystallography.

1. The Improbability of Discovery of "Linear" Active Regions by Cleavage Methods.

> "Sure it is easy to take a bunny apart but lets see you try and put one together again."[8]

The inhibitory region of Tn-I indicates that there are at least three factors that work against the discovery of "linear" active regions by cleavage methods.

Firstly, of all the species of Tn-I sequenced to date only rabbit fast Tn-I has two methionines arranged so as to closely bracket the inhibitory site. In other species of Tn-I the loss of one or both these methionines results in fragments approximately one hundred residues in length. Yet it has been shown that at least one peptide of this size that contains the inhibitory site has substantially lower activity than the twenty-two residue cyanogen bromide inhibitory fragment (Syska et al., 1976). If rabbit fast Tn-I had been missing one of these crucial methionines the low activity of the fragment might have caused the inhibitory site to have gone unnoticed or been deemed unimportant. Thus it may be that many potential "linear" active regions are never found because the proteolytic fragments that contain them are too large.

If some cleavage methods generate fragments that are too large, the Tn-I inhibitory region predicts that some generate fragments that have no activity because portions of the active site are missing. As evidence of this consider the difficulty of using digestive enzymes that have specificity for aromatic, basic or hydrophobic side chains to make a fragment that would contain an intact inhibitory site from Tn-I when these residues are all found within the inhibitory region. Enzymes with

[8] The author, Often, 1975-1981.

these specificities are the most commonly used in cleavage studies. There has even been a proposal, which will be covered in the section on X-ray crystallography, that active regions will be especially vulnerable to proteolytic cleavage.

Finally as we have demonstrated any fragment of a protein must be treated as an analogue since new charges have been introduced by the cleavage. These charges may reduce the activity to such an extent that the assay for an active fragment would not discover them.

If the above factors are important it may be that "linear" active regions may not be as rare in proteins as they are in cleavage fragments.

2. The Improbability of Discovery of "Linear" Active Regions by X-ray Crystallography

One of the most striking features of the inhibitory region of Tn-I is its composition. Of the 10 amino acids involved, five are basic, three are hydrophobic and two are proline. We have demonstrated the importance of two of the basic residues (lys^{105}, arg^{113}) and one of the hydrophobes (val^{114}) but the overall impression that remains is that the peptide is very highly charged. The importance of the residues mentioned implies that the complementary binding site contains two carboxyls and a hydrophobe so it seems possible it is highly charged (albeit) negatively. What is the significance, if any, of these charged groups?

Williams (1979) has pointed out that proteins that have hydrophobe to charged amino acid ratios of less than 2 tend; to form random coils, to be structural or control proteins rather than enzymes, and they tend to be difficult to crystallize without cofactors. The hydrophobe to charge ratio for cardiac Tn-I for example is 1.05.

The proposal states that the large proportion of charged residues is responsible for great chain mobility of these proteins which results in low amounts of secondary structure and the difficulty in crystallization. The proposal further states that if these charges are localized in a portion of the sequence this would lead to the formation of a very mobile loop which could fit indentations in another protein, much like fingers in a glove. The antigenic regions of some proteins, which are after all nothing more than protein-protein interaction sites, have been shown in some cases to be short peptide sequences that contain all of the antigenic activity and have many charged residues (Atassi and Lee, 1978).

A few consequences of these proposals are critical to a discussion of the seeming rarity of "linear" active regions. Firstly the active regions that are "three dimensional", i.e. composed of residues widely separated by the sequence but conformationally adjacent, are most likely to be found on proteins stable enough to preserve the conformation necessary for activity. These proteins are also the ones that will be easiest to crystallize and therefore are disproportionately represented in the group of proteins whose structure has been solved crystallographically. Conversely, proteins with low hydrophobe to charge ratios are least likely to have structure. In this kind of protein one might expect that "linear" active regions would be most likely because of the greater improbability of bringing residues widely separated by the sequence together when both portions of the chain of which they are part, are highly mobile. These kind of proteins are less likely to form crystals and consequently probably are under represented among those proteins whose structure has been solved crystallographically.

Secondly if the active regions themselves are highly charged their mobility ensures that they are accessible to attack by cleavage methods, especially proteolytic cleavage. This relates to our earlier discussion and indicates that it may be the "linear" active regions that are the most vulnerable to cleavage within the region and the consequent loss of activity by the resulting fragments.

3. Some Examples of Other "Linear" Active Regions of Proteins

In spite of the outlined difficulties inherent in locating linear interacting sequences some have been found that inhibit Ca^{2+} binding (Leavis et al., 1978; Reid et al., 1980) and antigenic determinancy (Atassi, 1975; Atassi and Lee, 1978). The antigenic determinant story has an interesting twist. While some of the determinants were composed of linear sequences others were composed of two linear regions sequentially distant but conformationally adjacent. These were made adjacent by disulfide bridges connecting the two regions and so from at least one perspective they were linear albeit through a disulfide link rather than the α-carbon chain. The twist came because Atassi and Lee (1978) were able to duplicate the antigenic site by synthesizing a peptide that simulates the surface with the two linear regions joined through their α-carbon chain by glycine residues. The overall effect being to remove all the residues between the cysteines which make the disulfide link and then replacing the disulfide link with two glycines.

A final piece of evidence that "linear" active regions may not be important only to solid phase synthetic chemists exists in the concepts of exons. It has been suggested that exons, regions of DNA which are expressed flanked by regions called introns which are not, code for amino acid sequences that have specific functions (Gilbert, 1978; Tonegawa et

al., 1978). A protein may consist of several exons and thus new proteins could be constructed from the functional domains that were involved in the assembly of old ones. These linear sequences of DNA that code for linear sequences of amino acids that my form functional domains need not be large as evidenced by the exons coding for the hinge region of the immunoglobulin heavy chain, coding for 14 residues (Sakano et al., 1979) and the signal peptide of the immunoglobulin light chain which contains the information coding for 19 residues (Tonegawa et al., 1978).

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